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U.S. National Institute of Health

TREASURY DEPARTMENT.

Public Health and Marine-Hospital Service of the United States.

WALTER WYMAN, Surgeon-General.

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HYGIENIC LABORATORY.—BULLETIN No. 21.

APRIL, 1905.

THE IMMUNITY UNIT

Biological
& Medical
Serials

FOR STANDARDIZING

DIPHTHERIA ANTITOXIN

(BASED ON EHRLICH'S NORMAL SERUM).

Official Standard prepared under the Act approved July 1, 1902.

BY

M. J. ROSENAU,

DIRECTOR OF THE HYGIENIC LABORATORY.

WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1905.

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The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress, March 3, 1901.

The following *bulletins* [Bulls. Nos. 1-7, 1900 to 1902, Hyg. Lab., U. S. Mar.-Hosp. Serv., Wash.] have been issued:

No. 1.—Preliminary note on the viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

No. 3.—Sulphur dioxide as a germicidal agent. By H. D. Geddings.

No. 4.—Viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 5.—An investigation of a pathogenic microbe (*B. typhi murium* Danyz) applied to the destruction of rats. By M. J. Rosenau.

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No. 7.—Laboratory technique: Ring test for indol, by S. B. Grubbs and Edward Francis; Collodium sacs, by S. B. Grubbs and Edward Francis; Microphotography with simple apparatus, by H. B. Parker.

By act of Congress, approved July 1, 1902, the name of the "United States Marine-Hospital Service" was changed to the "Public Health and Marine-Hospital Service of the United States," and three new divisions were added to the Hygienic Laboratory.

Since the change of name of the Service the bulletins of the Hygienic Laboratory have been continued in the same numerical order, as follows:

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No. 16.—The antiseptic and germicidal properties of glycerin. By M. J. Rosenau.

No. 17.—Illustrated key to the trematode parasites of man. By Ch. Wardell Stiles.

No. 18.—An account of the tapeworms of the genus *Hymenolepis* parasitic in man, including reports of several new cases of the dwarf tapeworm (*H. nana*) in the United States. By Brayton H. Ransom.

(Continued on third page of cover.)

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PREFACE.

The object of this bulletin is to describe the methods by which the immunity unit for measuring the strength of diphtheria antitoxin is obtained, and the principles involved.

The unit is based on the one established by Ehrlich, and has been made by comparison with the normal serum sent to this laboratory by the Kgl. Pr. Institut für experimentelle Therapie, Frankfurt a. M., Germany.

On account of the technical difficulties met in testing the strength of diphtheria antitoxin, and the intricate composition of the diphtheria poison, as well as the peculiar chemical relations existing between the toxine and the antitoxin, it has been necessary to enter into the many details of every portion of the process in order that those who use this standard may do so under the same conditions. Otherwise comparable results would not be obtained.

In such an apparently simple procedure as measuring two pieces of metal, as is done in duplicating the standard meter and yard, the greatest attention to many details must be observed. It can therefore be well understood how important it is, in comparing a standard based upon the physiological action of a mixture of two complicated organic substances, not to neglect the smallest detail that would insure accuracy.

As the unit made in the Hygienic Laboratory, U. S. Public Health and Marine-Hospital Service, is the legal unit for this country, the methods by which it is produced, as described in this bulletin, may be taken as official.

All standards are fundamentally arbitrary quantities. If all the meter bars or yardsticks were lost it would be difficult if not impossible to produce others having the exact lengths of the originals, which are therefore guarded against harm under conditions of uniform temperature, etc., to prevent changes in length.

The antidiphtheritic serum that has been carefully standardized and is used as the unit of strength for the measurement of diphtheria antitoxin is similarly kept under strict conditions of light, heat, moisture, etc., in the Hygienic Laboratory, U. S. Public Health and

Marine-Hospital Service. From time to time duplicates of this serum will be made to guard against deterioration or accident to the original.

This standard serum is preserved in small tubes. Every two months one of these tubes is opened, tested, and distributed to the licensed manufacturers and others who are working in this line.

The particular object of this standard is to insure the strength of antidiphtheritic serum sold in the United States by licensed manufacturers.

In accordance with the act of Congress approved July 1, 1902, no one is allowed to engage in interstate traffic in antitoxin without a license issued by the Secretary of the Treasury on recommendation of the Surgeon-General of the Public Health and Marine-Hospital Service. This license is issued only after a careful inspection of the establishment, its methods of manufacture, and an examination of its products for purity and potency at the hygienic laboratory of this Service.

The strength of diphtheria antitoxin is expressed in units. As the question has often been asked, What is the unit for this country? it became the duty of the Public Health and Marine-Hospital Service to establish and maintain a standard of measurement in order to bring about uniformity among American manufacturers. There is also the advantage of guaranteeing to physicians and patients using this wonderful specific remedy for diphtheria the fact that they are employing a remedy of sufficient strength to protect or cure. Weak serums have been shown to be practically useless.

The very unfavorable results obtained in England in 1895 in the handling of diphtheria with serum were due, according to the report of the Lancet Commission (Lancet, July 19, 1896), in a great majority of cases to the use of serums too weak to produce therapeutic results.

Experimental researches, for example, the very careful work of Madsen, in Copenhagen, 1896, showed that for therapeutic purposes weak serum is of very little value.

The fact need not be emphasized, as Ehrlich pointed out long ago, that for the whole question of the cure and prophylaxis of dipththeria by a serum therapy, as well as for the purposes of scientific investigation, it is important to use an antitoxin whose value has been definitely ascertained.

ACKNOWLEDGMENT.

As this standard is based upon the one established by Ehrlich, the principles involved and the methods employed are practically a reproduction of his work. First of all, I have to acknowledge not only the debt I individually owe, but the debt that all the world owes, to the remarkable genius of Ehrlich, who has done more, perhaps, to forward the problems of serum therapy than anyone else, not excepting Behring and Roux, the discoverers of the practical uses of diphtheria antitoxin. It is therefore evident that in the following pages no claim to originality can be made. In a few instances some improvements in the methods of testing, leading to increased accuracy, have been introduced. I have naturally drawn heavily upon the published literature on the subject, particularly the writings of Ehrlich, also Morgenroth, von Dungern, Madsen, Arrhenius, Marx, Smith, and Welch.

A bibliography of all the articles consulted in writing this bulletin, as well as in the work of standardizing the serum, will be found on page 85.

It is a pleasure to acknowledge particularly the assistance I have had from my colleague, Dr. Reid Hunt, Chief of the Division of Pharmacology, whose deep insight into physiological chemistry has helped me to a better understanding of some of the problems involved in making the immunity unit.

I also wish to acknowledge the assistance especially of Passed Assistant Surgeons John F. Anderson, the assistant director in this laboratory, and also Passed Assistant Surgeon R. von Ezdorf, and T. B. McClintic, and Assistant Surgeons Edward Francis and A. M. Stimson, who have faithfully carried out many details of the work under my supervision.

The inception of the law and the benefits that have already resulted from its administration are properly credited to the Surgeon-General of the Public Health and Marine-Hospital Service. It is therefore a pleasant duty to acknowledge this as one of the many debts our country owes to the public health work of Walter Wyman.

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THE IMMUNITY UNIT FOR STANDARDIZING DIPHTHERIA ANTITOXIN.

[Based on Ehrlich's normal serum. Official standard prepared under the act approved July 1, 1902.]

By Milton J. Rosenau,

Passed Assistant Surgeon, Director Hygienic Laboratory, Public Health and Marine-Hospital Service.

EHRLICH'S SIDE-CHAIN THEORY OF IMMUNITY.

Ehrlich's[a] side-chain theory is a brilliant chemical conception, giving the only satisfactory explanation of many of the phenomena concerned in immunity. In one sense it has been likened to Weigert's teachings of inflammation and the process of repair in so far that cognizance is taken of nature's prodigality. For instance, a much larger amount of material is thrown out than necessary to repair a wound. So, too, in antitoxic immunity a much larger amount of antitoxin is produced than necessary to neutralize the toxine.

In Ehrlich's conception the fundamental processes of immunity reside in the cells of the body. These cells are attacked by the poison and if not destroyed are stimulated into the overproduction of "antibodies" capable of combining with and neutralizing the poison.

Just what cells of the body play the most important rôle in the production of immunity is not exactly clear. It may be, as Ehrlich supposes, that this power resides in any organ or tissue.

In Metchnikoff's[b] theory of phagocytosis the free ameboid cells of the body play the all-important rôle. It is the macrophages and microphages seeking food that engulf and destroy the bacteria. In the process of digesting the bacteria the cell secretes the enzymelike substance which has the power of neutralizing the bacterial poisons.

It is surprising that the nutrition and metabolism of the cell lie at the foundation of two such divergent theories as Metchnikoff's and Ehrlich's.

---

[a] Ehrlich: Die werthbemessung des diphtherieheilserums und deren theoretische grundlagen. Klin. jahrb., Jena, v. 6 (2), 1897, pp. 299–326.

——— Ueber die constitution des diphtheriegiftes. Deut. med. woch., Leipzig, v. 24 (38), 1898, pp. 597–600.

——— Croonian lecture. On immunity with special reference to cell life. Proc. roy. soc., London, v. 66, pp. 424–448, pls. 6–7.

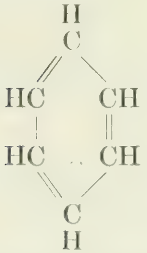
[b] Metchnikoff, Elie: L'immunite dans les maladies infectieuses. Paris, 1901. 45 figs., 600 pp.

According to the former it is the physical movements of the ameboid cell searching for food which, by a process known as phagocytosis, takes the bacteria into its protoplasm, forms a digestive vacuole around it, and then excretes the enzymelike substances (cytase[a]) useful in protecting the organism against the soluble poison of the bacteria.

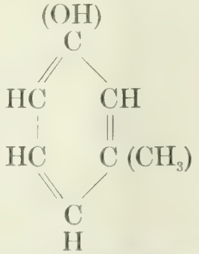
According to Ehrlich the hungry protoplasm of any cell, with its complicated molecule having side chains of various combining affinities ready to unite with suitable food molecules brought to it by the blood and body juices, lies at the foundation of his explanation of the chemical production of the antitoxin. It is strange that the same combining affinity should exist between the protoplasm of the cell and the proteid molecules that furnish it food as between the cell protoplasm and the toxines[b] of the bacterial poisons.

In considering Ehrlich's[c] side-chain theory it is necessary to disregard the microscopic structure of the cell and to think of the protoplasm as consisting of living molecules of extraordinary chemical complexity. The molecule of protoplasm has a central "nucleus" with "side chains," "lateral chains," or "bonds" of varying combining capacities. These "side chains" serve to bind the molecule to other molecules having proper combining affinities.

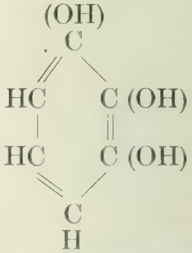
This arrangement of molecules with side chains is a well-known occurrence in organic compounds. The benzol ring forms one of the best and simplest examples.



Benzol C_6H_6



Metacresol $C_6H_4(CH_3)(OH)$



Pyrogallic acid $C_6H_3(OH)_3$

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[a] Macrocytase, microcytase, philocytase.

[b] I have made a distinction in this bulletin between "toxine" and "*toxin*." Some confusion arises in the literature for want of two words to represent two separate substances. The diphtheria poison contained in the toxic broth was first called "toxine," usually "toxin." It is now often spoken of as the "diphtheria poison." This filtered broth, containing a number of poisonous substances, I have called the toxine.

I have restricted the name "*toxin*" to the most important constituent of the toxine. The *toxin*, as will be found later, is only one of several allied poisons found in the toxine.

[c] Ehrlich: Die wertbemessung des diphtherieheilserums und deren theoretische grundlagen. Klin. jahrb., Jena, v. 6 (2), 1897, pp. 299-326.

By replacing one of the H atoms in the benzol ring with the methyl radical (CH_3) we have toluol; by replacing one of the H atoms with the hydroxyl group (OH) we have phenol; by substituting two hydroxyl groups we have resorcin, etc.; three, pyrogallic acid, etc.; by substituting one hydrogen atom of the ring with the hydroxyl radical and another one with the methyl radical we have the cresols.

These simple illustrations from well known organic compounds illustrate the central molecular mass of atoms with its side chains and combining affinities, to which the molecule of protoplasm is likened.

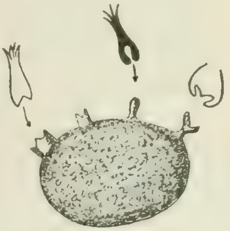

Fig. 1*a*.*—The cell with its various combining groups or side chains, known as receptors. Various toxines are shown having specific affinity for the proper shaped receptors.

In applying this analogy to the molecule of protoplasm the name "receptor" is given these side chains, or secondary atomic complexes of the molecular group. Contrary to the simple analogies above given each molecule of protoplasm has many different kinds of receptors, as shown by the schematic diagram in fig. 1*a*. These receptors have a specific affinity for the molecules of food, and also combine with the toxic molecules.

The *toxin* molecule, according to Ehrlich, consists of two important parts. One is known as the *toxophore group*, the other as the *haptophore group*.

The *toxophore group* of the toxin is that portion of the molecule which exerts a poisonous effect upon the protoplasm of the cell. This group is less stable than the haptophore group.

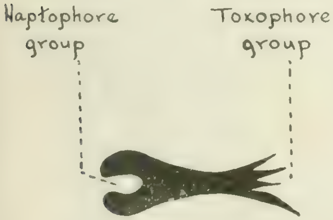

Fig. 1*b*.—The *toxin* molecule; showing the haptophore (combining) group, and the toxophore (poison) group.

The *haptophore group* is the seizing or combining portion of the *toxin* molecule (ἅπτω, to seize or attack). The haptophore group of the *toxins* have specific combining affinities for the receptors of certain cells, which in part explains the selective action of these poisons.

Toxines, such as diphtheria toxine, gradually diminish in toxicity, but retain the same power of chemical combination with the antitoxin. This phenomenon is explained by the formation of *toxoids*.

Ehrlich inferred the presence of the *toxoid* from the following simple experiment: He had a toxine which required 0.003 c. c. to kill a guinea pig. After nine months this poison weakened, so that it required three times as much; that is, 0.009 c. c. to kill a guinea pig. Nevertheless, the combining power of the toxine for antitoxin remained the same.

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* Foot Note.—Fig. 1*a*–*i*.—Diagrammatic representation of Ehrlich's side chain theory of immunity (Croonian Lecture, Proc. Royal Society of London, vol. 66, 1900, p. 437).

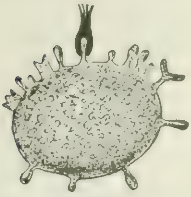

FIG. 1*c*.—The first stage of antitoxin formation; a *toxin* molecule anchored to a receptor.

Toxoids are altered *toxins*. They consist of the toxic molecule in which the toxophore group has been destroyed, leaving only the haptophore or combining group, which while able to satisfy the combining affinities of the antitoxin is no longer able to poison the protoplasm of the cell.

The diphtheria bacillus, during the process of its growth and multiplication in the body or in an artificial culture medium, produces several poisons, one of which is known as the diphtheria *toxin*. As above stated, the diphtheria *toxin* consists of a toxophore and haptophore group. In the body the latter unites chemically with the receptors of the cells. When this takes place one of two consequences may result: Either (1) the cell is so severely poisoned that it dies, or (2) the living molecule of protoplasm is stimulated so as to excite a defensive action by the reproduction of the receptors. Continued stimulation produced by the periodical injection of toxine results in an over production of receptors which finally loosen and float free in the blood serum and body juices.

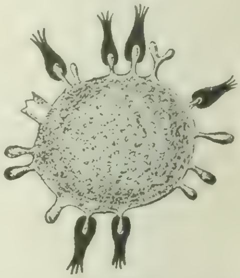

FIG. 1*d*.—The second stage; continued stimulation causes a reproduction of receptors.

Antitoxin consists of these free receptors floating in the blood serum. If now we introduce a liquid containing the *toxin* into the blood, it is immediately neutralized by combining with the free receptors through its haptophore group. All the combining affinities of the *toxin* are thus satisfied or saturated, so that the *toxin* is no longer able to unite with the receptors still attached to the cell, and this poison is thus rendered harmless.

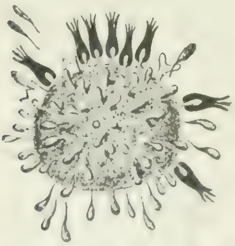

FIG. 1*e*.—Third stage: the receptors beginning to leave the cell.

It is of the greatest practical importance to know from what organs or tissues the receptors derive their origin.

It is by no means a necessary corollary of the side chain theory, as is often supposed, that the receptors are found only in those organs upon which the poisonous effects of a toxine are particularly manifested. On the contrary, Ehlich and Morgenroth[a] believe that receptors capable of combining with the toxine are produced in many different parts of the body, especially in tissues and organs having the power of anchoring the toxine without causing serious poisonous effects.

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[a] Erhlich, P., & Morgenroth, J.: Wirkung und entstehung der aktiven stoffe im serum nach der seitenkettentheorie. Handbuch der pathogen mikroorganismen, W. Kolle and A. Wassermann, Jena, 1904.

The connective tissue is believed to be especially rich in receptors, evidenced by the local reaction caused by the subcutaneous inoculation of diphtheria toxine, ricin, abrin, and similar poisons. In fact, one would not be far wrong in assigning a particular significance, in the production of receptors, to just those organs which show unimportant vital response, because in such tissues the injurious effects of the toxophore group are absent or of such diminished importance that the regenerative powers of such tissues are not retarded.

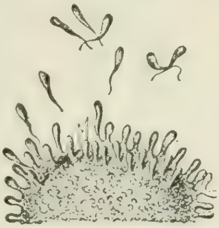

FIG. 1*f*.—Fourth stage: the receptors have left the cell and float free in the blood = antitoxin.

The presence or absence of receptors capable of binding the toxine, as well as their number and distribution, are factors which determine the susceptibility of different species of animals against the various toxines. These factors also determine the individual variations in the susceptibility to poisons and further explain the instances of natural immunity to toxines.

An example is given by Sachs,[a] who studied the reaction of guinea-pig blood against "arachnolysin," a toxine found in spiders. In this case the complete immunity of the red blood cells of the guinea pig against arachnolysin is accounted for by the entire absence of the proper receptors, while the susceptibility of the red blood cells of the rabbit to very small quantities of this poison is accounted for by the strong combining affinity which exists between these cells of the rabbit and the arachnolysin.

In some cases the production of receptors may apparently be traced in the development of certain species. Cannus and Gley[b] have followed the development (?) of the receptors in the red blood cells of the rabbit toward the hemolysin found in eel serum. Young rabbits are much less susceptible to this poison than adult rabbits, which is accounted for by Ehrlich as being due to a gradual development of the receptors having proper combining affinities for the hemolysin found in the eel serum.

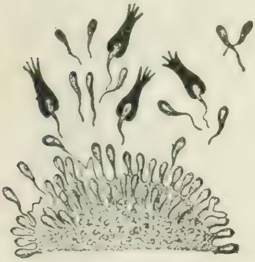

FIG. 1*g*.—The neutralization of a *toxin* by antitoxin: the free receptors in the blood have united with the *toxin* = antitoxic immunity.

The union between the receptor of the cell and its poison is not always a direct one, as described above, but sometimes takes place through the intervention of a second body, known variously as the amboceptor, zwichenkörper, immune body, sensitizer, fixative, preparative, desmon, etc.

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[a] Sachs, Hans: Hofmeisters Beitr., bd. 2, h. 1–3.

[b] Quoted by Ehrlich, loc. cit.

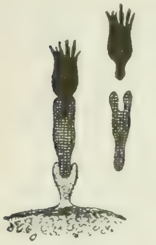

FIG. 1*h*.—The second order of immunity, showing the complement and immune body.

This order of immunity is particularly evident in the poisons that have a lytic or dissolving action upon bacteria or the cells of the body, such as the bacteriolysins, hemolysins, and other cytolysins. These poisonous bodies are believed by Buchner to be a ferment, and are usually spoken of as the "complement," but also as the "alexin" (Buchner) or "cytase" (Metchnikoff).

One of the remarkable facts connected with the phenomena of the lytic poisons is that the poison itself, usually called the complement, is normally present in the blood. The complement has less resistance to heat than the intermediary body, and is therefore spoken of as being thermolabile.

According to Ehrlich's theory, immunity can only be obtained against the intermediary body, which is believed to be specific. This intermediary body has a greater resistance to heat than the complement; that is, it is relatively thermostabile.

Ehrlich compares the intermediary body with diazo-benzaldehyde, which by means of its diazo group is capable of combining with a series of bodies, such as aromatic amins, phenols, keto-methyl bodies, etc., while by means of its aldehyde group it may combine with a different series, such as the hydrazins, ammonia radicals, and hydrocyanic acid. Phenol and hydrocyanic acid will not directly combine, but with diazo-benzaldehyde acting as an intermediary body, these two substances can be brought into combination. Pushing this comparison further, we may say that the aromatic body, or the phenol, represents a constituent of the blood corpuscle. The diazo-benzaldehyde is the intermediary body, while the poisonous hydrocyanic acid constitutes the complement.[a]

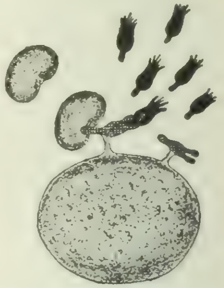

FIG. 1*i*.—The third order of immunity, showing an immune body having two affinities.

Welch[b] very ingeniously extended Ehrlich's conception of immunity to the bacterial cell. According to Welch's views the bacterial cell has the same power of defensive action against the poisons produced by the cells of higher animals that they have against the toxic products of the bacteria.

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[a] Vaughan and Novy: Cellular toxins, 1902, p. 131.

[b] Welch, William H.: The Huxley lecture on recent studies of immunity with special reference to their bearing on pathology. Bull. Johns Hopkins Hosp., Balto., v. 13, (141), Dec., 1902, pp. 285-299.

In other words, there is a chemical battle. Both the bacterial cell and the body cell excrete poisonous substances against each other, and both in turn are building up a chemical defense against the action of these respective poisons.

THE IMMUNITY UNIT.

The unit for measuring the strength of diphtheria antitoxin established by Ehrlich is a measure of strength, not of quantity.

It is difficult to define the unit in a brief sentence. A proper understanding of it may only be had from a study of the theoretical considerations involved.

The unit may be defined as the neutralizing power possessed by an arbitrary quantity of diphtheria antitoxic serum kept under special conditions to prevent deterioration in an authorized laboratory.

From a theoretical view point the unit may be defined as that quantity of diphtheria antitoxic serum which will just neutralize 200 minimal lethal doses of a pure poison. By a "pure" poison is understood one containing only *toxin*, and no *toxoid*, *toxone*, or other substances capable of uniting with the antibodies.

The test by which the strength of antitoxin contained in a unit is measured is a physiological one and depends upon the neutralization of the toxine by the antitoxin. This neutralization can only be determined by injecting the toxine and antitoxin mixtures into guinea pigs, which animals are highly susceptible to the diphtheria bacillus and its poisons.

In order to obtain a fundamental understanding of Ehrlich's immunity unit it is necessary to make a study of the nature of the various poisonous substances which are present in the diphtheria toxine, and then clearly to understand the combining and neutralizing action which these poisons exert upon the antitoxin. Both of these subjects are treated more fully in the next chapter.

In all the earlier work on this subject the toxine was used as a basis for measuring the strength of the antitoxin, but as the toxine is a much more complex substance than the antitoxin, and as it is less stable, accurate results were not possible. Ehrlich showed that the antitoxin under certain conditions was permanent, both in power of chemically combining with and physiologically neutralizing the toxine.

The unit for measuring the strength of diphtheria antitoxin is an arbitrary quantity, just as the units of all systems of weights and measures are fundamentally arbitrary quantities, so that the question of the quantity of immunity units which a particular diphtheria antitoxic serum contains can only be determined by comparing it with the standard serum preserved by Ehrlich in the Klg. Institut für Experimentelle Therapie at Frankfort a. M., Germany. This serum has been carefully tested, its antitoxic strength is accurately known, and by comparing any other serum with this original one its antitoxic value may be determined.

Ehrlich, however, does not admit that his antitoxic unit is such an arbitrary quantity, and believes that he can reproduce it should it become lost, by a study of the peculiar relations which exist between the combining and neutralizing power of toxines and antitoxins; for, from a theoretical point of view, Ehrlich[a] considers that the immunity unit contains just 200 "combining units." A combining unit is analagous to the valence in chemistry, and Ehrlich has shown by his study of various diphtheria poisons that the antibodies in his immunity unit have 200 of these combining affinities. As each combining affinity represents one minimal lethal dose, the immunity unit theoretically should combine with and neutralize 200 MLD's of a toxine containing only *toxin*. If it were possible to obtain the *toxin* in pure solution, free from *toxoids*, *toxones*, and other substances which have the power of uniting with the antibodies, it would then be comparatively easy to demonstrate the number of minimal lethal doses which the immunity unit is able to neutralize. As such pure poisons are practically never obtained, the immunity unit in actual practice is found to neutralize many times less than 200 minimal lethal doses of the toxine (from 16 to 136), the remaining combining affinities being satisfied by such allied substances as *toxoid*, *toxone*, etc., which have the same chemical affinity for the antitoxin, but diminished poisonous properties.

In order the better to understand Ehrlich's immunity unit it will be necessary to give a brief review of its development. In all the earlier systems of measuring the potency of diphtheria antitoxic serum the diphtheria culture or its toxine was the basis of measurement. Behring and Roux both first used living cultures. Behring substituted the use of the soluble diphtheria poison for living cultures as early as 1893, as he found it was impossible to establish any system of definite dosage with live organisms.

The method at first used by Roux for determining the strength of antitoxic serum was based upon the ratio between the quantity of the serum necessary to protect a guinea pig and the weight of that guinea pig. His method was carried out as follows: A certain quantity of diphtheria antitoxic serum, say 0.01 c. c., was injected subcutaneously into a guinea pig. Twelve hours later, 0.5 c. c. of a fresh virulent culture of the diphtheria bacillus was inoculated into the guinea pig, weighing, say, 300 grams. If the guinea pig lived, 0.01 c. c. of the serum was considered sufficiently active to protect 300 grams of guinea pig, and 1 c. c. of the serum would consequently protect 30,000 grams of guinea pig, and the strength of the serum was expressed as 1: 30,000. The strength of a serum according to this method was expressed in such figures as 1: 100,000 or 1: 500,000.

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[a] Ehrlich: Die wertbemessung des diphtherieheilserums und deren theoretische grundlagen. Klin. jahrb., Jena, v. 6 (2), 1897, pp. 299–326.

Ehrlich: Ueber die constitution des diphtheriegiftes. Deut. med. woch., Leipzig, v. 24 (38), Sept. 22, 1898, pp. 597–600.

Ehrlich: Ueber die giftcomponenten des diphtherietoxins. Berl. klin. woch., 1903, nos. 35–37, p. 37.

Control animals were inoculated to insure the virulence of the culture used. The culture used was considered sufficiently virulent if the control guinea pig died within thirty hours.

A serum with an immunizing strength of 1: 100,000 meant that 1 c. c. would protect 100,000 grams of guinea pig; and a serum whose strength was 1: 500,000 meant that 1 c. c. of the serum would protect 500,000 grams of guinea pig against a certainly lethal dose of diphtheria culture or later the toxine when injected in accordance with the above procedure.

Madsen[a] showed as a result of comparative tests that the German method of expressing the strength of antitoxic sera in antitoxin units was quicker, cheaper, easier, and more accurate than the French method devised by Roux. He showed that with the French method it is often impossible to determine differences as great as 1: 100,000 and 1: 200,000. This method was finally abandoned by Roux himself in favor of the German procedure.

In the earlier methods of testing the strength of diphtheria antitoxin, according to both the methods of Behring and Roux, the two substances were always inoculated separately into a guinea pig, usually in different places. Sometimes the toxine and antitoxin were inoculated at different times in order to make a distinction between the immunizing power of a serum and its curative power.

The method devised by Ehrlich in collaboration with Kossel and Wassermann in 1894[b] was based upon an entirely new principle. The scientific researches of Behring and Kitasato had previously established the fact that tetanus poison and its antitoxin neutralize each other in a test tube outside of the body. Ehrlich, Kossel, and Wassermann satisfied themselves that this neutralization between the diphtheria poison and its antibody takes place at once when the two substances are mixed in a test tube, and they believed at that time (1894) that it took place in accordance with the law of simple proportions.

[a] Madsen, Thorvald: Ueber messung der stärke des antidiphtherischen serums. Zeitschr. f. hyg., Leipzig, v. 24, pp. 425–442.

[b] Ueber Gewinnung und Verwendung des Diphtherieheilserums. Deut. med. Woch., 1894, no. 16.

In the methods used before this time (1894), when the toxine and antitoxin were injected separately into the guinea pig the substances first met each other in the body of the animal. The time it takes for the toxine to meet and neutralize the antitoxin when these substances are injected separately into an animal naturally varies with the individual conditions of absorption, and exact results therefore were not possible. It was found, on the other hand, that if the two substances are mixed in a test tube and the mixture is injected into guinea pigs, constant and uniform results are obtained.

At this time (1894) an old test toxine, obtained by Behring and preserved with 0.5 per cent phenol, was used as a basis for measuring the strength of the antitoxic serums. Ten times the minimal fatal dose of this toxine for a guinea pig weighing 200 to 300 grams was mixed with varying quantities of serum the strength of which was to be determined. The mixture was at once inoculated into guinea pigs and the animals closely watched for local reaction at the site of inoculation, for loss of weight or other symptoms. As the symptoms appear in a few days it was possible, in carrying out the tests according to this method, to determine the strength of an antitoxin in a short time.

This is Behring's method of measuring the strength of diphtheria antitoxic serum.

The strength of the serum was determined in accordance with the tests carried out by Behring's method, as that quantity of serum which completely neutralized the ten minimal fatal doses. The toxine was considered to be completely neutralized if the guinea pig showed neither local nor constitutional effects of any kind. At that time (1894) Ehrlich considered the method as outlined above to be trustworthy, and established an ANTITOXIN UNIT as that quantity of serum which required just 1 c. c. to neutralize ten times the minimal fatal dose of the diphtheria poison. The antitoxic serum, which had precisely this power, he considered as a normal serum.

Later von Behring and Ehrlich found stronger serums and modified the unit, which they now call the immunity unit, to be that quantity of antitoxin, viz, antidiphtheritic serum, which will neutralize 100× MLD for a guinea pig weighing 250 grams. Ehrlich's method for carrying out the tests in accordance with this new conception was as follows:

Ten times the minimal fatal dose is mixed with a definite quantity of serum and this mixture injected subcutaneously into a guinea pig. If the guinea pig lives the quantity of the serum used contains at least one-tenth of an immunity unit.

For example, if the minimal fatal dose of toxine is 0.005, ten times this quantity, or 0.05, represents ten times the minimal lethal dose (10 × MLD).

The proper quantity of the toxine and an amount of the antitoxin to be tested are now mixed in a test tube, as follows: To 0.05 c. c. of the toxine is added 0.001 c. c. of the serum, and the mixture inoculated subcutaneously into a guinea pig. If there was sufficient antitoxin in the 0.001 c. c. of the serum used to neutralize the 10 MLD's used, the pig would survive, and it was then considered that 0.001 c. c. of that serum contained at least one-tenth of an immunity unit, and that 1 c. c. of that serum would consequently contain at least 100 units.

Attention is particularly called to the fact that at first the strength of antitoxin depended upon the complete neutralization of the poison as determined by indications of sickness in the guinea pig, particularly changes at the site of inoculation. Slight swellings which disappear in a few days were not taken into consideration in determining the value of the serum. It is evident that the indications of sickness in the guinea pig or the presence of slight changes at the site of inoculation are dependent upon subjective considerations leading to differences of opinion or errors in judgment which could materially affect the accuracy of the tests.

A partial neutralization of the poison sufficient to save the life of the animal is a more definite factor than the complete neutralization depending upon the appearance of local signs for recognition.

On account of these difficulties encountered in determining the value of antitoxins it became necessary to eliminate all subjective considerations and obtain a method based upon strictly objective conditions. Both Ehrlich and von Behring independently came to the conclusion that the *death* of the animal was a much better criterion for determining the value of a serum than the local reactions or the survival of the animal heretofore depended upon.

A still more radical change in the principle of measuring the strength of diphtheria antitoxin was now introduced.

Up to this point the basis of measuring the strength of diphtheria antitoxin was always either the culture of the diphtheria bacillus or its poisonous products. The genius of Ehrlich disclosed errors in the methods heretofore described. His researches into the biological relations between toxine and antitoxin showed that it required varying amounts of antitoxin in order to neutralize the 10 MLD's. He finally showed that there is no relation between the poisonous effects of the toxine and its power of combining chemically to neutralize the antitoxin. He showed that a toxine may be weakened very materially by the influence of time, light, heat, oxygen, and other deleterious influences, without, however, altering its power of combining with antitoxin. This discovery was of fundamental importance and required the transfer of the standard of measurement from the poison to its antibody.

Ehrlich[a] now made numerous researches in order to determine the stability of the antitoxin. He had particular success with a serum containing a high percentage of glycerin. This glycerinated serum showed exactly the same power of neutralizing toxine after one year that it did at first.

For a while this glycerin solution of antitoxic serum was used as the basis for standardizing serums. But Ehrlich showed that even glycerinated serum may weaken and also the fact that because the neutralization point does not vary is no guaranty of the stability of the test solution. It was therefore necessary to abandon the glycerinated serum as a method for preserving the standard unit. The principal factors which cause the weakening of antitoxin are moisture, oxygen, light, and heat.

It is very easy to guard against the deleterious influences of light and heat. It was therefore necessary only to pay particular attention to the first two agents.

The serum was therefore reduced to a powder under proper precautions and this dried blood serum preserved in a small glass apparatus consisting of two parts connected by means of a glass tube. The serum is placed in one portion of the apparatus and phosphoric anhydrid, which is a very powerful dehydrating substance, in the other. The air is exhausted as much as possible by means of a high vacuum and the apparatus hermetically sealed. After a few days the acid will have taken up all the moisture from the serum, and the tube connecting the two parts may then be melted and sealed off, separating the acid from the serum. The dried serum is now contained in a vacuum tube and preserved in a cool place in absolute darkness.

In this way Ehrlich prepared a great number of these little tubes, each one containing a dried serum whose value was accurately determined. Each tube contained 2 grams of the dried serum, representing 1,700 immunity units in each gram. Every two or three months one of these tubes is carefully opened and the contents dissolved in 200 c. c. of a 10 per cent solution of sodium chlorid and glycerin mixture, the mixture containing from 50 to 80 per cent of glycerin. One c. c. of this glycerinated serum represents then exactly seventeen times the normal strength; and therefore if we dilute 1 c. c. of this glycerinated serum with 16 parts of water, each cubic centimeter of the dilution contains one immunity unit.

Although the serum is used as a basis for standardizing the strengths of other sera, the toxine still plays a very important rôle in the process of testing, as it is manifestly impossible to estimate the strength of an antitoxic serum by comparing it directly with another serum. One is compared with the other through the toxine.

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[a] Ehrlich: Die Wertbemessung des Diphtherieheilserums und deren theoretische Grundlagen. Klin. Jahrb., Jena, v. 6 (2), 1897, pp. 299–326.

It is not the absolute toxicity of the poison which is considered in testing the strength of the antitoxin, but a combination of its combining and poisonous powers, as expressed in the L+ dose (see p. 26). The method by which the strength of standard serum is estimated and methods by which other serums are compared with it are fully considered in detail in the following pages.

It is to be noted here that in accordance with the method of determining the power of diptheria antitoxin, as devised by Ehrlich, three important factors are achieved: The poison is neutralized, and the protective and curative powers of the serum established.

THE CONSTITUTION OF THE TOXINE AND ITS RELATION TO ANTITOXIN.

Ehrlich's discovery that the relation between toxine and antitoxin is not always the same led him to investigate the constitution of the diphtheria poison.

It is believed that the diphtheria bacillus primarily secretes at least two poisons, the *toxin* and the *toxone.*

The *toxin* is not stable and is readily reduced to *toxoid.* For the purposes of our work we must have a clear understanding of the *toxin*, the *toxoid*, and the *toxone.*

The *toxin* is the only poison produced by the diphtheria bacillus capable of causing acute death. The *toxoids* have little or no poisonous properties. The *toxones* produce the late manifestations of paralysis.

The interesting point about all of these poisons is, they have the power of combining chemically with the antitoxin. In other words, *toxin*, *toxoid*, and *toxone*, and finally *epitoxonoid*, all have the same haptophore group, but different or modified toxophores.

The poisonous *toxins* are subdivided into three groups depending upon the degree of their avidity for antitoxin, viz., *prototoxin*, *deutrotoxin*, and *tritotoxin.* Each of these *toxin* groups may in whole or in part be converted into *toxoids*, which are not poisonous but which have the same power of combining with the antitoxin as the *toxin* from which they derive their origin. These altered *toxins* are consequently known as *prototoxoid*, *deutrotoxoid*, and *tritotoxoid*, respectively. The *prototoxin* has a greater affinity for antitoxin than the *deutrotoxin*, and the *deutrotoxin* has a greater affinity for antitoxin than the *tritotoxin.* The same relation holds good for the three *toxoids.*

As each diphtheria poison contains these substances in varying proportions it is at once evident that there can be no relation between the toxicity of the poison and its combining power with antitoxin. Take for example two poisons which require the same dose to kill, but in one the *prototoxin* and in the other the *tritotoxin* has become altered into *prototoxoid* and *tritotoxoid*, respectively. It is then evident that in order to neutralize the same lethal dose in the first case it would require more serum than in the second, because in the first case a certain proportion of the antitoxin would be used up by combining with the *prototoxoid*, which is nonpoisonous, leaving a proportionately greater amount of uncombined *toxin.* On the other hand, a serum tested against ten or one hundred minimal lethal doses of these respective poisons would appear to have quite different immunizing values.

The *toxone* is one of the primary metabolic products of the diphtheria bacillus. It possesses the same haptophore group as the *toxin*, but has far less avidity for antitoxin. When the diphtheria poison and antitoxin are brought together the *toxone* is the last to enter into combination with the antitoxin, as both the *toxin* and *toxoid* have greater affinities for the antibodies than the *toxone*. The *toxone*, as before mentioned, has the same haptophore group as the *toxin* but has a different toxophore group, as it is incapable of producing acute effects, cutaneous necroses and death, but is responsible for the local edema and the diphtheritic paralysis.

The diphtheria poison is first investigated biologically by adding varying quantities of the poison to the immunity unit and inoculating the mixtures into a series of guinea pigs. In this way it is very evident, as Ehrlich has shown, that two limits, boundaries, or zones are always shown, which are of the greatest importance in determining the nature and composition of the poison. Each of these limits is designated by the letter L, from *limes*, a boundary or zone.

These limits are known, respectively, as L^0 (^{0}=nil) and L+ (+ = death).

By L^0 is meant that quantity of poison which just neutralizes or saturates one immunity unit as shown at the necropsy done forty-eight hours after the subcutaneous injection of the mixture into the guinea pig. The reaction at the site of the inoculation at this examination must be hardly noticeable.

Theoretically the L^0 dose of toxine must unite with and neutralize just 200 "combining units" of antitoxin. The L^0 dose, therefore, contains just 200 minimal lethal doses of a theoretically pure poison.

By L+ is meant the smallest quantity of toxine that will neutralize one immunity unit, plus a quantity necessary to kill the animal on the fourth day. As defined by Ehrlich, the L+ dose is that quantity of poison which, despite the antibodies contained in one immunity unit of serum, contains a sufficient excess of the poison to cause the death of the guinea pig within the course of four days.

In studying the constitution of the diphtheria poison it is also necessary to determine its absolute toxicity with the greatest possible precision. The absolute toxicity of the diphtheria poison is usually spoken of as the minimal lethal dose (MLD), sometimes as the minimal fatal dose (MFD), and occasionally as the simple lethal dose. The determination of the minimal lethal dose is often an exceedingly tedious problem, and in some cases requires as many as 100 animals. This is due in part to the fact that the determination of the exact limits is largely influenced by the individuality of the guinea pigs, so that it is necessary to repeat the work on a series of animals in order to reach an average.

The minimal lethal dose may be defined as that quantity of toxine which will surely kill every guinea pig weighing 250 grams in the course of four days or, at the very latest, five days. As Ehrlich has pointed out, such a quantity may kill some of the animals sooner, that is, within thirty-six to forty-eight hours. A quantity of diphtheria poison that will kill every guinea pig, without exception, acutely, within thirty-six to forty-eight hours, contains more than the minimal lethal dose.

The L+ dose is a much more definite and constant factor than the MLD. It does not show the irregularities or present the difficulties met with in determining the absolute toxicity of the poison.

In his earlier work Ehrlich looked upon the diphtheria toxine and antitoxin as simple substances neutralizing each other as an alkali does an acid. That the relation between these two substances is much more complex was at once evident as soon as he worked out the complex nature of the diphtheria poison.

The best method of studying the relations between toxine and antitoxin is by mixing these two substances together in varying amounts and studying the effects of partial saturation or neutralization. As the immunity unit is supposed to contain 200 "combining units," the most valuable information is obtained by adding one two-hundredths of the immunity unit to a given quantity of toxine, using either the L^0 or L+ dose of toxine. These mixtures, inoculated into a series of guinea pigs of standard weight and under standard conditions, will give certain definite results.

By mixing with the L^0 dose of the diphtheria toxine such fractional amounts of antitoxin we have results which may be summarized as follows:

At first these mixtures may show no diminution in toxicity, despite the addition of considerable (say, forty two-hundredths) of the immunity unit. This zone represents the *prototoxoids*. Then comes a time when for each addition of one two-hundredth of a unit of antitoxin the poison may lose one minimal lethal dose. This is the zone of *toxins* (*syntoxins*). Then follows the zone in which the mixture fails to produce acute death, but may cause local edema and later paralysis. This is the zone of *toxones*, first called *epitoxones* by Ehrlich.

Later, von Dungern[a] showed the presence of *epitoxonoids* which have the same combining affinity for the antitoxin, but show no poisonous properties. The *epitoxonoids* of von Dungern explain the power of neutral mixtures of toxine and antitoxin to produce immunity when injected into susceptible animals.

The results of the partial saturation or neutralization tests are conveniently studied by plotting curves, called by Ehrlich "spectra." Several such spectra are shown in fig. 2.

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[a] Beitrag zur kenntniss der bindungsverhaltnisse bei der vereinigung von diphtheriegift und antiserum. Deut. med. woch., v. 30 (8–9), 1904, pp. 275–277, 310–312.

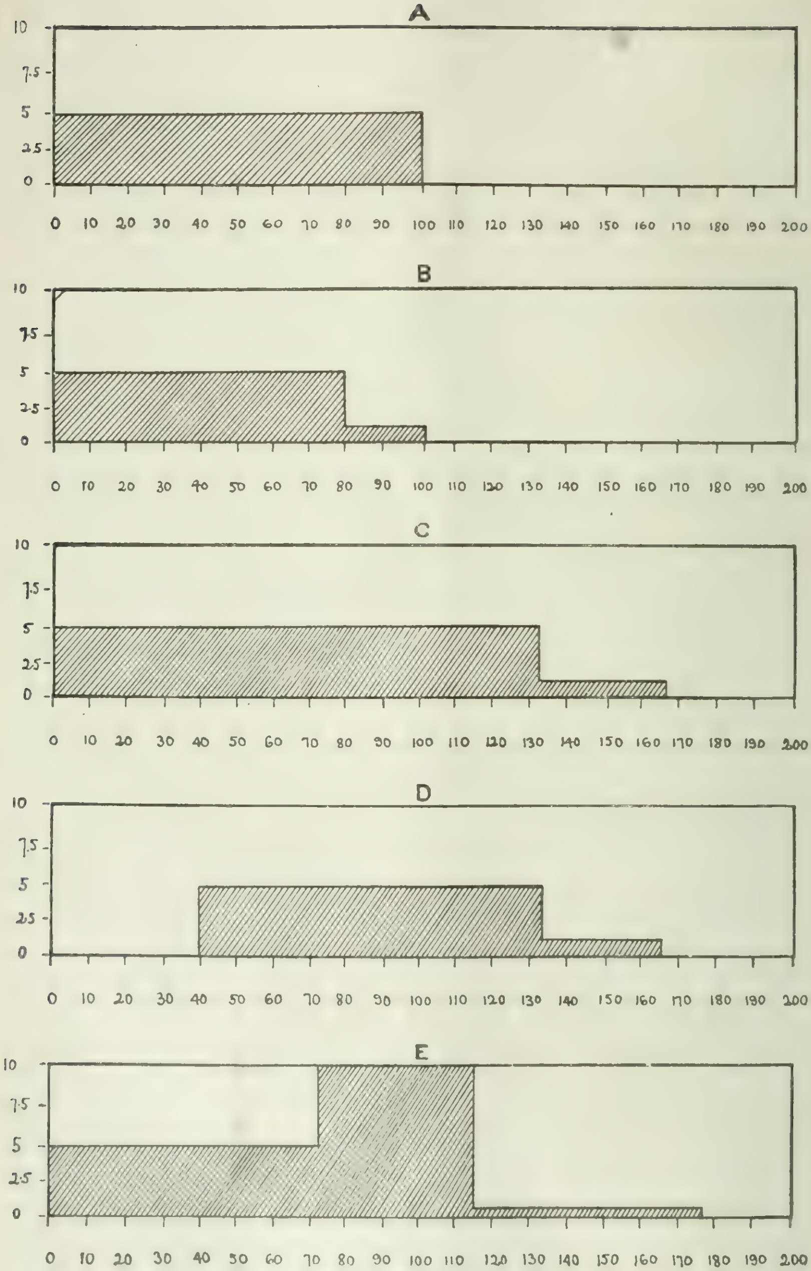

FIG. 2 — Ehrlich's spectra.

In studying these spectra, it must be remembered that one immunity unit theoretically neutralizes 200 minimal lethal doses of a pure poison, containing only *toxin*. It is self-evident that such *toxin* "valences" may be substituted by other substances having the same combining affinity but less poisonous or not at all poisonous, such as *toxoid* and *toxone.*

As toxines containing only *toxin* are practically never obtained, it is evident that the immunity unit will be found in actual practice to neutralize a less number of minimal lethal doses than 200, the remaining number being satisfied with the *toxoids* and *toxones.*

In order to obtain a correct understanding of these spectra, it is necessary to know how one is cast. The most instructive results are obtained by using the L^0 dose of toxine. The L^0 dose is that quantity of toxine which will just neutralize one immunity unit. The L^0 dose should contain just 200 MLD's of a theoretically pure toxine, containing only *toxin.*

Let us make a spectrum of an impure toxine containing the following:

$L^0 =$ 50 combining units of *prototoxin.*
100 combining units of *toxin* (=100 MLD's).
50 combining units of *toxone.*
—
200

Then:

L^0 dose of toxine = 100 MLD's, i. e., contains enough *toxin* to kill 100 guinea pigs.
$L^0 + \frac{1}{200}$ immunity unit = 100 MLD's.
$L^0 + \frac{49}{200}$ immunity unit = 100 MLD's.
$L^0 + \frac{50}{200}$ immunity unit = 100 MLD's.

Up to this point we see that the addition of fifty two-hundredths of the immunity unit has no effect upon the toxicity (*toxin*) of the poison. In other words, the antitoxin is being used up by combining with some nonpoisonous substance, viz, *prototoxoid.* The first 50 segments of our spectra will therefore consist of this modified *toxin.*

If now we add more antitoxin to our toxine we will find that for the addition of each one two-hundredths of the immunity unit the toxine loses one MLD—

$L^0 + \frac{51}{200}$ immunity unit = 99 MLD's.
$L^0 + \frac{70}{200}$ immunity unit = 80 MLD's.
$L^0 + \frac{147}{200}$ immunity unit = 3 MLD's.
$L^0 + \frac{148}{200}$ immunity unit = 2 MLD's.
$L^0 + \frac{149}{200}$ immunity unit = 1 MLD.

That is, the L^0 dose of toxine plus one hundred and forty-nine two-hundredths of the immunity unit contains in the mixture just enough free poison (*toxin*) to kill one guinea pig. Therefore the next 100 segments of this spectrum will consist of *toxin*.

If we now add one two-hundredths more of antitoxin the mixture will fail to kill. Thus,

$L^0 + \frac{150}{200}$ immunity unit — will not kill acutely, but causes paralysis (*toxones*).

$L^0 + \frac{197}{200}$ immunity unit — will not kill acutely, but causes paralysis (*toxones*).

$L^0 + \frac{198}{200}$ immunity unit = will not kill acutely, but causes paralysis (*toxones*).

$L^0 + \frac{200}{200}$ immunity unit = complete neutralization.

The last 50 segments of the spectrum would, therefore, be made up of *toxone*.

The spectrum of this poison would, therefore, be expressed as follows:

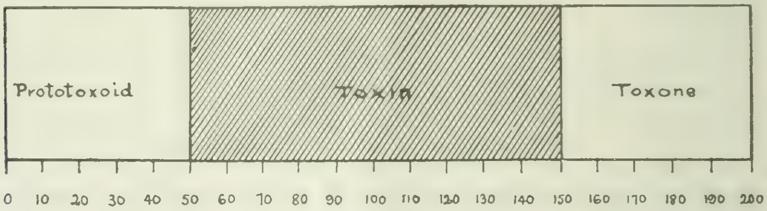

FIG. 3.—A spectrum of a simple poison. Redrawn from Madsen, La Constitution du poison diphtherique. Ann. de l'Institut Pasteur. Vol. XIII, 1899, p. 576.

By this graphic representation of the results of the studies of toxine-antitoxin mixtures it is possible to tell at a glance the proportional amounts of *toxin*, *toxoid*, or *toxone* contained in a given poison.

The following instructive instance is taken from Ehrlich's poison No. 5:[a]

L^0 dose of this toxine No. 5 was 0.125 cc.
L+ dose of this toxine No. 5 was 0.25 cc.
MLD dose of this toxine No. 5 was 0.0025 cc.

It will, therefore, be seen that the L^0 dose of this particular toxine contained exactly 50 × MLD, and the L+ dose contained 100 × MLD.

The gradual changes which this poison underwent, as shown by the spectra, may be explained as follows:

In A (fig. 2) pure *hemitoxin* is shown. By this is meant that the addition of each one two hundredths of the immunity unit to the L^0

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[a] Ehrlich: Ueber die constitution des diphtheriegiftes. Deut. med. woch., Leipzig, v. 24 (38), 1898, pp. 597–600.

Ehrlich: Ueber die giftcomponenten des diphtherietoxins. Berl. klin. woch., 1903, nos. 35–37.

dose takes away just one-half MLD until 100 such combining units, or one-half of the immunity unit, has been added. A further addition of antitoxin had no effect upon the *toxin* as shown by death or necroses, but only influenced the *toxone.*

In B (fig. 2), instead of an equal mixture of *toxin* and *toxoid*, known as *hemitoxin*, we have a mixture of *toxin* and *toxoid*, containing more of the latter than the former, as indicated by the diminished toxicity. The evidence of *tritotoxin* is shown in the spectra between 80 and 100.

Phase D (fig. 2) shows a further loss of toxicity of this poison No. 5 under consideration. By this time the minimal lethal dose had risen to 0.004. It will be seen that the addition of forty two-hundredths of the immunity unit had no influence upon the toxicity of the poison, indicating the appearance of *prototoxoid.*

Three distinct zones may be distinguished in such spectra.

The first zone, which is not always present, represents the non-poisonous *prototoxoid.*

The second zone contains the poisonous *toxin.*

If the addition of $\frac{1}{200}$ of the immunity unit, which represents one combining unit, removes exactly 1 MLD, the poison then contains pure *toxin.* Sometimes the addition of one combining unit removes on the average one-half the lethal dose. This phenomenon is interpreted as indicating the mixture of equal parts of *toxins* and *toxoids*, designated by Ehrlich as *hemitoxin.* The existence of *hemitoxin* is possible only in case the *toxoid* has precisely the same avidity for the antitoxin as the *toxin.* From this it follows, as Ehrlich had from the beginning assumed and as Arrhenius has confirmed, that in the transformation of *toxin* into *toxoid* no change in avidity occurs.

Pure *toxoid* occurs often in the form of a *prototoxoid*, which possesses a stronger affinity for antitoxin than all other components of the poison. The *prototoxoid* manifests its presence by the fact that a certain quantity of antitoxin may be added to the diphtheria poison without in the slightest degree lessening its toxicity.

The third zone, which Ehrlich calls the "toxone zone," has been subject to the greatest amount of discussion and the correctness of his views has been questioned, especially by Arrhenius and Madsen.[a] Arrhenius insists that instead of considering the diphtheria poison to contain *toxin* and *toxone*, it would be simpler to consider it as a single (homogeneous) substance which has a very weak affinity for the antitoxin, and that in mixtures containing toxine and antitoxin there is always both free toxine and free antitoxin. He draws his analogy from known facts in physical chemistry, particularly from studies upon

[a] Toxines et antitoxines le poison diphtérique. Acad. roy. sci. et let. Danemark: Bull. no. 4, 1904. pp. 269–305.

the relation of boracic acid and ammonia. These two substances have a comparatively weak affinity for each other, and in mixtures all the boracic acid does not combine with the ammonia, but there is always present both free ammonia and free boracic acid.

When ammonia and boracic acid are brought together in watery solution some of the ammonia at once unites with some of the boracic acid and forms ammonium borate. This reaction starts with a certain velocity, but as the mass of ammonium borate increases, the velocity of the reaction gradually diminishes. After a time a condition is reached when the ammonium borate has a maximum value and does not further increase, no matter how long the reaction is allowed to proceed under the given conditions.

When this condition of equilibrium is reached the mass contains a constant quantity of water, ammonia, boracic acid, and ammonium borate; but these substances are not at rest. The ammonia and boracic acid will always react when in the presence of each other whether or not ammonium borate is present. But as the proportionate amount of ammonium borate remains constant, it is understood that while this continuous association between the ammonia and the boracic acid is going on there is, at the same time, a reversible action—that is, a dissociation of the ammonium borate to re-form ammonia and boracic acid. These two reactions take place simultaneously.

Arrhenius believes that the diphtheria poison changes slowly, according to the laws of monomolecular reactions, into a nonpoisonous body—toxoid. Both substances, toxin and toxoid, according to Arrhenius, combine feebly with antitoxin, the equilibrium constant being equal for both.

Ehrlich however, contends on the other hand that the diphtheria poison is not only a complex substance, but that the *toxin* and antitoxin have strong affinities for each other. He admits that the long interval between the values of L^0 and L + seems to oppose the acceptance of a strong affinity between the *toxin* and antitoxin.

Designate by D the amount of toxine representing the difference between L + and L^0. From chemical examples it can be easily shown that with poisons of strong avidity the value of D must correspond exactly to one minimal lethal dose, whereas with poisons of weak affinity D may be much larger on account of the free or dissociated poison. Ehrlich, however, finally succeeded in finding a toxine in which D was precisely of the theoretic value of 1. Thereby it was in principle shown that *toxin* and antitoxin unite with strong affinity, and the great variation, from 0 to 300 per cent, in the value of D represented by different specimens of toxines could be explained by the presence of *toxone*.

In his recent very careful work upon the constitution of diphtheria poison and the relation between toxine and antitoxin, von Dungern[a] concludes that—

1. The union of diphtheria toxine and antitoxin does not proceed in accordance with the ammonia-boric acid scheme.

2. The observed phenomena of combination are explicable only upon the assumption of a complex constitution of the diphtheria poison.

3. The facts find their best explanation in the action of a *toxone* and an *epitoxonoid*. *Epitoxonoid* is present in considerable quantity in toxic broth. The immunizing action of apparently completely neutralized toxines can therefore be explained.

4. Constituents of the diphtheria poison with weak affinity after combining with antitoxin may eventually become so firmly bound that this union can only be incompletely broken by toxin of stronger avidity. The strength of the combination is of significance for the action of antitoxin.

The claim of Arrhenius and Madsen that the toxine is a simple substance having a weak affinity for the antitoxin, and that the combination of toxine and antitoxin follows the Guldberg-Waage law, and that the reaction is therefore reversible, seems, in the light of the evidence before us, to be untenable.

Nernst[b] and Michaelis[c] consider that the assumption that the reaction is reversible, at least after a very short period, is arbitrary and unsupported by evidence. In addition to von Dungern,[a] as above quoted, Sachs,[d] Morgenroth,[e] and others have brought forward new experiments to show that the reaction is not reversible, and that the existence of *toxoids* and *toxones* is very probable.

The question as to the existence of *toxones* now seems by the work of Calcar, if confirmed, to be definitely settled in favor of Ehrlich's views. Calcar[f] separated from diphtheria bouillon by special methods of filtration, through a membrane, two poisonous constituents. The one (Ehrlich's *toxin*) killed acutely; the other (Ehrlich's *toxone*) produced late paralysis and death after several weeks.

Ehrlich and his co-workers had been able to show the existence of *toxones* only by more indirect methods (partial neutralization of toxine with antitoxin), and lack of direct proof of their existence gave Arrhenius and Madsen's contention that the *toxones* were only the *toxin* which was being slowly freed from the antitoxin-toxine combination a certain degree of plausibility.[a]

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[a] Deut. med. Woch., 1904, Nos. 8 and 9.

[b] Zeit. f. Elektrochem., 10, p. 377.

[c] Biochem. Centrlbl., 3, p. 1.

[d] Berl. klin. Woch., 41, p. 412.

[e] Ibid., 41, p. 526.

[f] Ibid., 41, p. 1028.

Calcar was also able to show that the *toxones* have a greater molecular volume than the *toxins*, but a smaller molecular volume than the proteids.

It therefore seems plain, as Ehrlich states, that his views furnish a far better explanation than those of Arrhenius of numerous facts in the domain of toxines, such as the process of spontaneous enfeeblement of *toxins*, their passage into a state of stability, the reduction of the *toxone* zone, and the capacity of completely neutralized poisons to produce antitoxin (Park) by means of von Dungern's *epitoxonoids*.

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[a] Reid Hunt in Gould's Amer. Yearbook of Med. and Surg., 1905, p. 649.

THE TOXINE.

PREPARATION OF THE TOXINE.

The culture.—For the preparation of the toxine we use a culture known as "Park's bacillus No. 8." This culture has now become famous in laboratories both in this country and abroad on account of its remarkable power of retaining its virulence and producing strong poisons. The culture was isolated in 1894 by Dr. Anna W. Williams, of the New York health department, and is sometimes known as the Park-Williams bacillus.

The best results are obtained by growing the organism as a surface growth in the special bouillon presently to be described. We carry it over from test tube to test tube every day, taking only the surface film with a flattened platinum needle.

The strongest poisons are obtained when the surface growth is heavy and the bouillon remains clear. A precipitate of the old and dead organisms always collects at the bottom of the tube.

The bouillon.—The bouillon is a special alkaline medium prepared from fresh lean beef freed of muscle sugar and all other sugars, and to which a small quantity of dextrose (glucose) is added, which seems to favor the production of a strong poison.

The bouillon is prepared largely in accordance with the instructions laid down by Theobald Smith in a paper entitled "The relations of dextrose to the production of toxin in bouillon cultures of the diphtheria bacillus."[a] This culture medium, which we call "Smith's bouillon," is prepared by us as follows:

Cut out all the fat and tendon of the beef. Pass through the meat grinder, catching all the expressed juice. Weigh and add twice the weight of water. Place in the cool room at 15° C. for twenty-four hours, then strain through cloth, pressing firmly. Weigh the amount of meat infusion thus obtained. Take the reaction and neutralize with sodium hydrate to 1.5 per cent acidity to phenol-phthalin in order that the colon bacillus, which is now planted in it, may grow well. Inoculate with a reliable culture of *B. coli communis*, using 10 c. c. of a twenty-four-hour-old bouillon culture for each liter of meat infusion. Grow at 37° C. for twenty-four hours. Add the white of one egg for each liter of infusion. Heat gently for twenty minutes to coagulate the albumin, and then filter while hot through paper. Weigh the amount of filtrate obtained and add sufficient water to make up the loss to the original amount. Take the reaction again and neutralize with sodium hydrate to an acidity of 0.5 per cent, then add 1 per cent peptone and one-half per cent sodium chlorid and 0.1 per cent dextrose. Heat again for twenty minutes in streaming steam in an open autoclav. Again take reaction, neutralize and repeat until the reaction of the infusion is just 0.5 per cent. The medium is now filtered through paper and filled into Fernbach flasks, test tubes, etc. These are sterilized in the autoclav at a temperature of 120° C. for twenty minutes.

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[a] Journ. Exper. Med., vol. 4, nos. 3–4, 1899.

The fermentation tubes prepared and sterilized without the addition of the 0.1 per cent of dextrose are now planted with a young culture of the colon bacillus and incubated forty-eight hours. If there is no muscle sugar present the growth stops sharply at the closed end without gas production and the reaction of the medium in the closed and open arms is about the same.

Test tubes are also filled and the final reaction is taken from the medium in one of the test tubes which were sterilized. The reaction is always found to rise about 0.1, due perhaps to the formation of acid salts by the heat of sterilization.

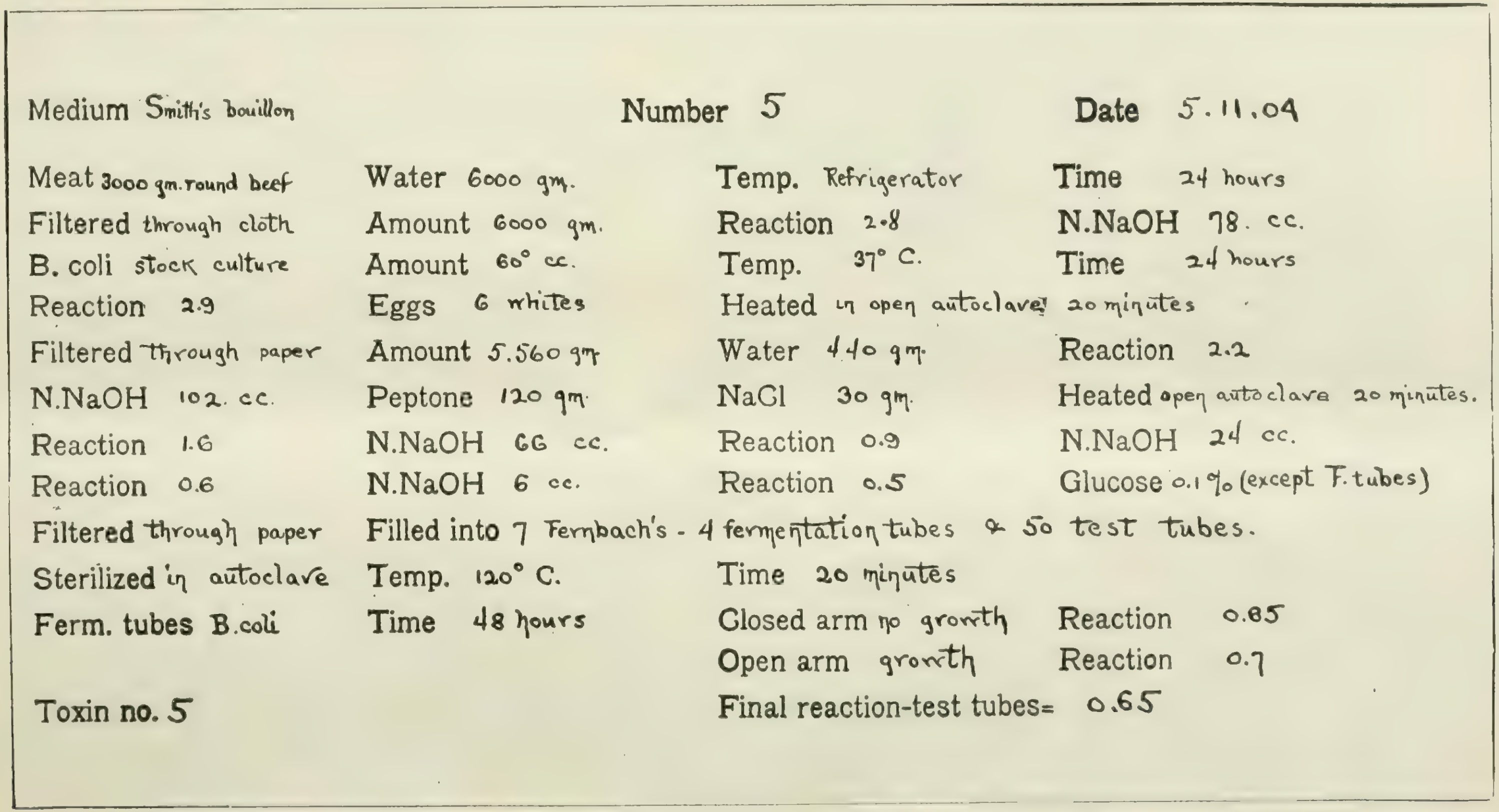
Our strongest toxines were obtained by adding the dextrose after the final sterilization and at the time of inoculation, confirming the results obtained by Hitchens working with Kinyoun.

The reaction of the bouillon used to grow the diphtheria culture is very important if we desire to obtain a strong poison. The reaction of the bouillon we use, 0.5 per cent acid to phenol-phthalin, is distinctly alkaline to litmus.

The diphtheria bacillus during the first period of its growth always produces an increased acidity in the bouillon. The culture used in this laboratory causes a marked rise in acidity during the first twenty-four or forty-eight hours and then a gradual change to alkali production, so that by the seventh day the reaction of the culture has returned to about its original point, 0.5 per cent.

Park and Williams, and also Theobald Smith, have shown that an excess of acid in the medium is detrimental to the production of strong poisons. The rise in acidity above noted amounts to 1 to 1.5 per cent. The reaction of the medium therefore never becomes more acid than 1.5 to 2 per cent, using phenol-phthalin as an indicator. A bouillon which is 1.5 per cent acid to phenol-phthalin is still distinctly alkaline to litmus.

The method as above described has given the best results in this laboratory. With it we have, in one instance, obtained a toxine so strong that 0.0008 c. c. was sufficient to kill standard weight guinea



Medium Smith's bouillon		Number 5	Date 5.11.04
Meat 3000 gm. round beef	Water 6000 gm.	Temp. Refrigerator	Time 24 hours
Filtered through cloth	Amount 6000 gm.	Reaction 2.8	N.NaOH 78. cc.
B. coli stock culture	Amount 60° cc.	Temp. 37° C.	Time 24 hours
Reaction 2.9	Eggs 6 whites	Heated in open autoclave 20 minutes	
Filtered through paper	Amount 5.560 gm	Water 4.40 gm.	Reaction 2.2
N.NaOH 102. cc.	Peptone 120 gm.	NaCl 30 gm.	Heated open autoclave 20 minutes.
Reaction 1.6	N.NaOH 66 cc.	Reaction 0.9	N.NaOH 24 cc.
Reaction 0.6	N.NaOH 6 cc.	Reaction 0.5	Glucose 0.1 % (except F. tubes)
Filtered through paper	Filled into 7 Fernbach's - 4 fermentation tubes & 50 test tubes.		
Sterilized in autoclave	Temp. 120° C.	Time 20 minutes	
Ferm. tubes B. coli	Time 48 hours	Closed arm no growth	Reaction 0.85
		Open arm growth	Reaction 0.7
Toxin no. 5		Final reaction-test tubes= 0.65	

Fig. 4.—Illustrating record kept of each lot of bouillon.

pigs within four days. We not infrequently obtain toxines, the MLD of which is 0.005 c. c. However, such favorable results are not always obtained. Sometimes for several weeks in succession our toxines have failed to kill guinea pigs in doses as large as 0.01 c. c.

It must be evident to anyone who has had practical experience with the manufacture of diphtheria antitoxin that there are factors concerned in the production of a strong poison that are not at all understood. It is sometimes discouraging to those who take especial pains to comply with all the requirements believed to favor the production of strong toxines to find that other laboratories who use simpler methods obtain, on the average, poisons of equal and sometimes superior toxicity.

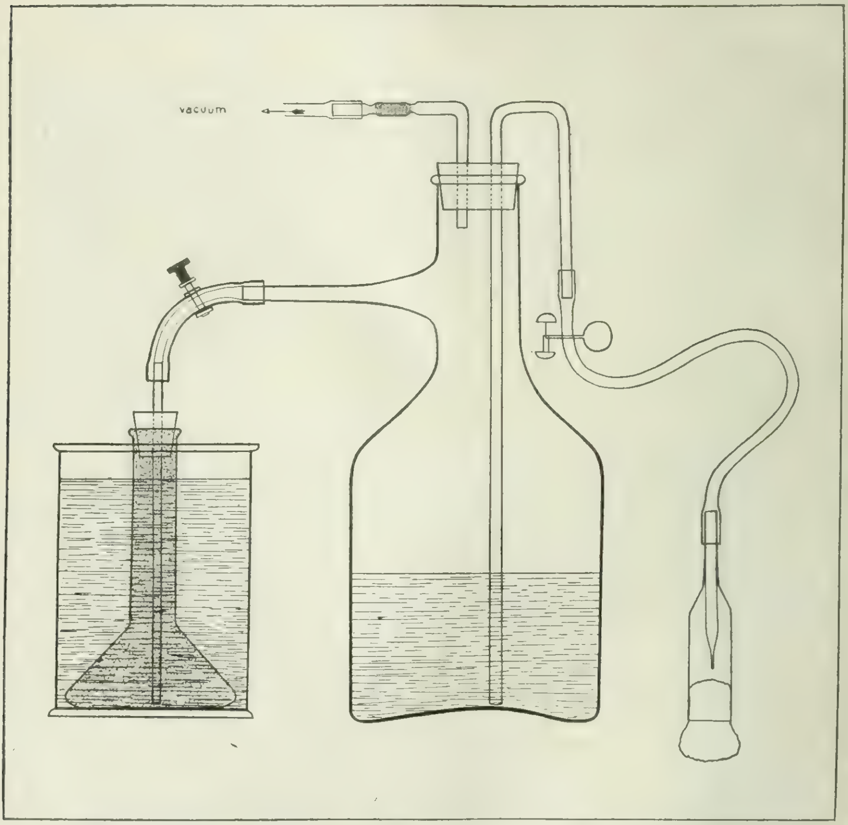


FIG. 5.—Method of filtering the culture. Flask for storing the toxine in bulk with Maasen mouth piece.

Roux and Yersin[a] noticed that the toxicity of a liquid increased more rapidly and more regularly when the culture is grown in the presence of air frequently renewed. They passed filtered air across the surface of the culture. This procedure, once widely employed, has now been generally abandoned, it having been found that it is sufficient to have a large surface of the culture exposed to air. It is now the universal practice in almost all laboratories to grow the toxine in shallow layers of broth contained in flat-bottomed vessels such as the Fernbach flask.

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[a] Macé, E.: Traité pratique de bactériologie. pp. 589–590.

Spronck[a] believed that the presence of glucose in the bouillon prevents the bacillus of diphtheria from producing strong toxic substances. He recommended, in order to eliminate the glucose, to use meat aged almost to the point of putrefaction. He also recommended that care should be taken to use a peptone freed from glucose and the addition to the bouillon of 0.5 per cent of sodium chlorid and a small quantity of carbonate of lime.

Nicolle[b] used fresh beef killed the same morning. He added 2 per cent peptone and 5 per cent salt.

Park and Williams[c] recommend the employment of a decidedly alkaline bouillon, containing 2 to 4 per cent peptone. They obtained the best results with a bouillon distinctly neutral to litmus and then an additional 7 c. c. of normal soda per liter. Such a bouillon has a distinct alkaline reaction to litmus.

Martin[d] insists that the boullion should be made with a fluid peptone obtained from the stomachs of hogs.

Spronck,[e] in his second process, reported success by growing the culture in a decoction of yeast.

In many laboratories a preference is given for the simplest methods, using an ordinary bouillon distinctly alkaline, following the recommendations of Park and Williams.

The methods used in this laboratory, as previously described, are largely taken from the experimental work of Theobald Smith.

Neutralization.—The reaction is taken with phenol-phthalin as an indicator as follows:

To 5 c. c. of the bouillon add 45 c. c. of distilled water plus 1 c. c. of a 0.2 per cent solution of phenol-phthalin in 95 per cent alcohol. Heat to boiling and titrate while nearly boiling with a $\frac{N}{20}$ solution of sodium hydrate. The reaction is indicated by the first appearance of a distinct rose-red color, best seen in a white porcelain dish before a well-lighted window.

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[a] Spronck: Sur les conditions dont dépend la production du poison dans les cultures diphtériques. Moyen simple de préparer une toxine très active. Ann. de l'Inst. Pasteur, IX, 1895, p. 758.

[b] Nicolle: Préparation de la toxine diphtérique. <Ann. de l'Inst. Pasteur, 10, 1896, p. 333.

[c] Park & Williams: The production of diphtheria toxin. <Journ. exper. med., I, 1896, p. 164.

[d] Martin: Production de la toxine diphtérique. <Ann. de l'Inst. Pasteur, 12, 1898, p. 26.

[e] Spronck: Préparation de la toxine diphtérique; suppression de l'emploi de la viande. <Ann. de l'Inst. Pasteur, 12, 1898, p. 701.

Example: 2.6 c. c. of the $\frac{N}{20}$ NaOH solution is found necessary to neutralize 5 c. c. of the bouillon in order to obtain the desired acidity of 0.5 per cent, add 2.1 c. c. (2.6−0.5=2.1), of the normal soda solution (40 to 1,000) to each 100 c. c. If an acidity of 1.5 per cent is desired, add 1.1 per 100 c. c. (2.6−1.5=1.1).

Each lot of bouillon is numbered and a complete record kept of every stage of the operation upon the blank shown in fig. 6.[a]

The bouillon is distributed in modified Fernbach flasks, presenting a large surface to the air for pellicle growth. Seven hundred and fifty c. c. of the bouillon is placed in each flask.

The freshly prepared media is inoculated upon the surface from a twenty-four-hour-old culture by means of a platinum spoon.

The flasks are incubated for seven days at 37.5° C.

They are then removed and examined for purity by means of cover slips. All atypical growths are discarded.

The final reaction of the culture when taken from the incubator is taken for each flask. This usually varies from 0.6 to 0.8 per cent. Very acid results, such as 1.5 or over, would, according to Smith's work, indicate the absence of a strong poison and may be discarded without further testing upon guinea pigs.

The bouillon is now filtered through a pear-shaped, unglazed porcelain filter or a Berkefeld candle by means of a vacuum. The arrangement for filtering the virus is shown on page 38. This diagram also illustrates the character of the flask used to store the toxine in bulk. It is very convenient to draw off small amounts from time to time by means of the siphon and Maasen nozzle without danger of contamination. The especially strong and otherwise suitable toxines are bottled from these flasks without the addition of any preservative.

Bottling and preserving.—The toxine is kept in bulk in the 2 liter bottle shaped flasks described above, and later divided into small ground-glass stoppered bottles holding 5 and 10 c. c. each.

The small bottles are filled to the neck with the toxine and the stopper inserted so that the air is all displaced and the fluid completely fills the vial.

A minute quantity of sterilized liquid petrolatum is touched to the ground glass of the stopper so as to prevent the two surfaces of glass

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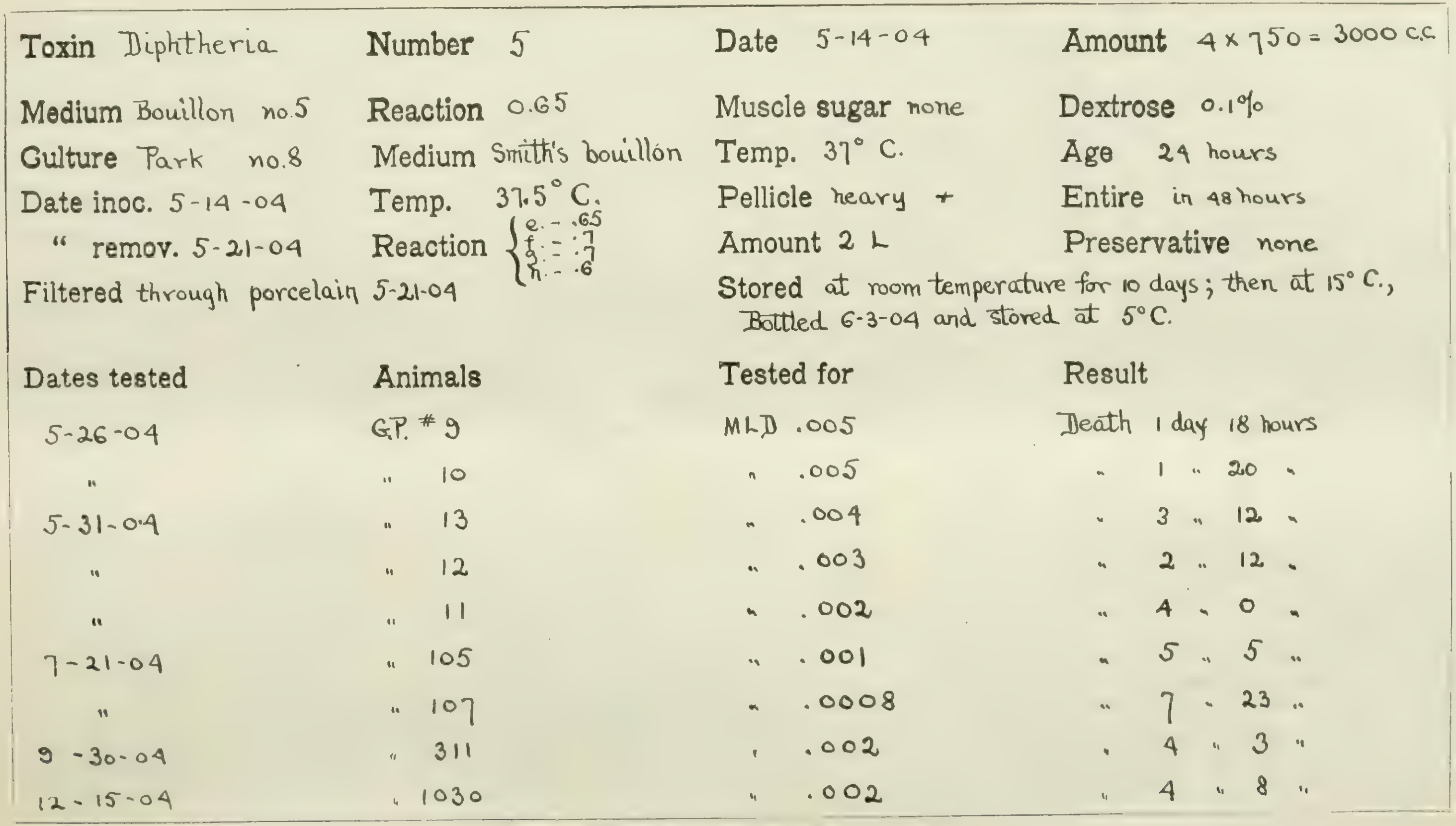
[a] I am indebted to Dr. Herbert D. Pease, director of the antitoxin laboratory, New York State department of health, for suggestions on the method of keeping records on the card system shown in figs. 4 and 6. Doctor Pease very kindly let me have blanks used by him, which were modified to suit our purposes.



Toxin Diphtheria	**Number** 5	**Date** 5-14-04	**Amount** 4 x 750 = 3000 c.c
Medium Bouillon no.5	**Reaction** 0.65	**Muscle sugar** none	**Dextrose** 0.1%
Culture Park no.8	**Medium** Smith's bouillon	**Temp.** 37° C.	**Age** 24 hours
Date inoc. 5-14-04	**Temp.** 37.5° C.	**Pellicle** heavy +	**Entire** in 48 hours
" **remov.** 5-21-04	**Reaction** { e. - .65 f. - .7 g. - .7 h. - .6	**Amount** 2 L	**Preservative** none
Filtered through porcelain 5-21-04		**Stored** at room temperature for 10 days; then at 15° C., Bottled 6-3-04 and stored at 5°C.	

Dates tested	Animals	Tested for	Result
5-26-04	G.P. # 9	MLD .005	Death 1 day 18 hours
"	" 10	" .005	" 1 " 20 "
5-31-04	" 13	" .004	" 3 " 12 "
"	" 12	" .003	" 2 " 12 "
"	" 11	" .002	" 4 " 0 "
7-21-04	" 105	" .001	" 5 " 5 "
"	" 107	" .0008	" 7 " 23 "
9-30-04	" 311	" .002	" 4 " 3 "
12-15-04	" 1030	" .002	" 4 " 8 "

FIG. 6.—Illustrating record kept of each toxine.

sticking to each other. As it is probable that some of these bottles will be kept for years, this may be an important detail. The neck of the bottle is carefully dried and then quickly dipped into melted paraffin with a high melting point so as to seal the joint completely and prevent evaporation or contamination.

Each bottle is labeled and stored in a special ice box kept at a constant temperature of 5° C.

It will be seen that according to this method the toxine is kept in absolute darkness, free from contact with the oxygen of the air, and at an equable and low temperature. By the use of the ordinary bacteriological precautions the addition of a preservative as toluol and similar substances is rendered unnecessary.

TESTING THE TOXINE.

It is first necessary to determine the minimal lethal dose of the toxine with approximate accuracy. Weak poisons are discarded. A fresh toxine requiring more than 0.01 c. c. to kill a 250-gram guinea pig is too weak for the purposes of this special work.

If the preliminary tests show that the poison has the required toxicity, its minimal lethal dose must then be accurately determined. This may require a number of animals.

After the absolute toxicity of the poison has been determined it is necessary to establish the L+ and the L^0 doses. An illustration of how these doses are obtained follows. These results are taken from our records.

Tests to determine the L+ dose of toxine No. 7.

	Result.
1 immunity unit + 0.19 c. c. toxine	Invariably causes late paralysis, never acute death.
1 immunity unit + .20 c. c. toxine	Sometimes causes late paralysis and sometimes acute death.
1 immunity unit + .21 c. c. toxine	Always causes acute death about the fourth day.
1 immunity unit + .22 c. c. toxine	Always causes acute death, usually on the second or third day.

The L+ dose of this toxine is, therefore, just 0.21 c. c.

Tests to determine the L^0 dose of toxine No. 7.

	Result at autopsy forty-eight hours after inoculation.
1 immunity unit + 0.14 c. c. toxine	No visible reaction.
1 immunity unit + .15 c. c. toxine	No visible reaction.
1 immunity unit + .16 c. c. toxine	Slightest possible congestion about carbon particles, or no reaction.
1 immunity unit + .17 c. c. toxine	Apparent reaction at site of inoculation.
1 immunity unit + .18 c. c. toxine	Injection and edema at site.
1 immunity unit + .19 c. c. toxine	Injection and edema at site.

The L^0 dose of this toxine, therefore, is 0.16 c. c.

These figures are actual results obtained with our toxine No. 7.

If the interval between the L+ and the L^0 dose is greater than 15 × MLD, the toxine contains too great a proportion of toxones, etc., and should not be used for testing the strength of antitoxin. If it were possible to obtain ideally pure toxines in which the interval between the L+ and the L^0 dose was only one minimal lethal dose, our problem would be simplified. Unfortunately such a poison has only once been demonstrated. However, by these preliminary tests upon a number of poisons we select the most suitable for the purposes of this special work.

Having selected a suitable poison, it is now laid aside to season. For this purpose the toxine should be kept in bulk, so that the process will be uniform throughout the mixture. If it is kept in diffused light at room temperature, the changes will take place quicker than if kept in the ice box. At intervals of about a month the toxine is tested in order to determine the rate of change. These examinations will disclose the fact that the toxicity gradually diminishes and the L+ dose increases. These alterations take place progressively until the poison arrives at a stage of equilibrium. Here it may remain for a long time. A short time before this stage of equilibrium is reached it is best to divide the toxine into small bottles, excluding the air, in order to guard against loss by accident or contamination, and store the entire lot in a cold place protected from light.

The toxine can not be depended upon for the purpose of testing the antitoxin until this stage of equilibrium is reached. After a varying length of time the toxine will again show change in toxicity, and when this second period of change sets in the toxine should be discarded as no longer serviceable for the purposes of these tests.

As soon as the toxine has reached its stage of stability or equilibrium, that is, when the test dose remains constant, it is necessary to determine this test dose (L+) with the greatest possible accuracy.

The well-standardized toxines used in this laboratory are tested against the immunity unit every week or two in order to assure ourselves that they are not undergoing change. A number of carefully tested toxines are always kept on hand, so that we may have a double test of the serums we are standardizing, as well as to guarantee against changes in the toxines which may set in suddenly.

The exact standardization of a toxine takes considerable time and we therefore can not depend upon one poison. The exact L+ dose of a well-seasoned toxine is the keynote of the whole testing operation. Without this test dose good results are impossible. Every effort must be made to obtain a reliable poison that answers all the requirements and, having such a toxine, to maintain it under the most careful conditions in order to keep it in a state of equilibrium as long as possible. As the toxine grows old the tests naturally multiply in number and increase its reliability for the purpose of determining the unit strength of antitoxins.

On account of the importance of the L+ dose I exhibit here a series of L+ tests showing the character of the results obtained by the poison after it has gained its stage of equilibrium.

Table giving the results of tests on Toxine No. 7 for L – dose, using 0.21 c. c. of the toxine against one immunity unit, from October 24, 1904, to December 22, 1904.

Guinea pig No.	Time of death.		Remarks.
	Dys.	*hrs.*	
395	3	0	
453	3	5	
454	5	15	
544	3	9	
545	3	21	
546	3	5	
547	3	18	
601	5	16	
602	2	17	Clear serous fluid in pleural cavities.
603	3	13	
604	3	6	
605	1	18	Omentum, mesentery, and broad ligament injected, etc.
621	3	2	
622	2	19	Clear fluid in pleural cavity.
623	3	2	
624	3	14	
625	3	7	
693	3	18	
694	4	11	
695	3	14	
696	4	8	
697	3	16	
750	3	19	
751	2	12	Peritoneum discolored and petechiæ throughout.
752	2	12	Some peritoneal reaction and clear serum in pleuræ.
753	2	14	Nothing unusual, adrenals congested, local reaction.
754	2	17	Do.
808	3	17	
809	4	14	
855	2	20	Usual reaction.
856	4	4	
857	3	9	
858	4	4	
859	2	20	Usual reactions.
860	2	15	Do.
903	3	7	
904	3	16	
905	6	2	
948	3	2	
949	3	2	
950	3	18	
951	3	18	
984	3	6	
985	1	18	Pneumonia.
986	1	17	Peritonitis; serous exudate; omentum very red.
987	5	6	
988	1	17	Pneumonia.
989	2	6	Clear serum in pleural cavities.
1063	4	20	
1064	3	0	
1065	3	12	
1066	4	13	
1067	3	8	
1068	3	4	
1069	1	17	Pneumonia, both upper lobes.
1124	2	17	Spleen and liver show sharply defined caseous areas.
1125	4	16	
1126	3	17	
1127	5	17	
1128	2	17	Usual reaction.
1129	3	6	
1150	3	8	
1151	1	19	Severe peritoneal reaction.
1152	2	17	Peritoneal fat injected.
1153	3	11	
1154	2	16	Clear serum in pleural cavities.
1155	2	12	Usual reaction.
1156	3	11	

During two months covered by the table every one of the 68 animals injected with 0.21 c. c. of this toxine, mixed with 1 immunity unit, died acutely. None recovered. Most of the guinea pigs died on the fourth day. As we usually inoculate our animals at about 3 o'clock in the afternoon, animals dying between three days two hours and four days two hours will appear on the autopsy table on the fourth day.

Animals dying before the fourth day usually show at autopsy some reason for this increase of susceptibility.

The following tables used for diluting our toxines for MLD and L+ doses will be found useful:

TABLES FOR DILUTING TOXINES FOR MLD.

First dilution.

1+99=1:100

Second dilution.

1 of first dilution+49=1: 5000	1 c. c.=.0002
	1.5 c. c.=.0003
	2 c. c.=.0004
	2.5 c. c.=.0005
	3 c. c.=.0006
1 of first dilution+39=1: 4000	0.8 c. c.=.0002
	1 c. c.=.00025
	1.2 c. c.=.0003
	1.6 c. c.=.0004
	2 c. c.=.0005
	2.4 c. c.=.0006
	2.8 c. c.=.0007
	3.2 c. c.=.0008
1 of first dilution+29=1: 3000	0.9 c. c.=.0003
	1 c. c.=.00033
	1.2 c. c.=.0004
	1.5 c. c.=.0005
	1.8 c. c.=.0006
	2.1 c. c.=.0007
	2.4 c. c.=.0008
	2.7 c. c.=.0009
	3 c. c.=.001
1 of first dilution+9=1:1000	1. c. c.=.001
	1.5 c. c.=.0015
	2 c. c.=.002
	2.5 c. c.=.0025
	3 c. c.=.003

TABLES FOR DILUTING TOXINES FOR MLD.—Continued.

First dilution.

1+49=1:50

Second dilution.

1 of first dilution+9=1:500 1 c. c.=.002
1.5 c. c.=.003
2 c. c.=.004
2.5 c. c.=.005
3 c. c.=.006

First dilution.

1+29=1:30

Second dilution.

1 of first dilution+9=1:300 0.9 c. c.=.003
1 c. c.=.0033
1.05 c. c.=.0035
1.2 c. c.=.004
1.35 c. c.=.0045
1.5 c. c.=.005
1.65 c. c.=.0055
1.8 c. c.=.006
1.95 c. c.=.0065
2.1 c. c.=.007
2.25 c. c.=.0075
2.4 c. c.=.008
2.55 c. c.=.0085
2.7 c. c.=.009
2.85 c. c.=.0095
3 c. c.=.01

1+99=1:100 1 c. c.=.01
1.5 c. c.=.015
2 c. c.=.02
2.5 c. c.=.025
3 c. c.=.03

1+49=1:50 1 c. c.=.02
1.5 c. c.=.03
2 c. c.=.04
2.5 c. c.=.05
3 c. c.=.06

TABLES FOR DILUTING TOXINES FOR MLD.—Continued.

1+29=1:30 .9 c. c.=.03
1.2 c. c.=.04
1.5 c. c.=.05
1.8 c. c.=.06
2.1 c. c.=.07
2.4 c. c.=.08
2.7 c. c.=.09
3 c. c.=.1

TABLES FOR DILUTING TOXINES FOR L+ DOSE.

1+19=1:20 1 c. c.=.05
1.1 c. c.=.055
1.2 c. c.=.06
1.3 c. c.=.065
1.4 c. c.=.07
1.5 c. c.=.075
1.6 c. c.=.08
1.7 c. c.=.085
1.8 c. c.=.09
1.9 c. c.=.095
2 c. c.=.1
2.1 c. c.=.105
2.2 c. c.=.11
2.3 c. c.=.115
2.4 c. c.=.12
2.5 c. c.=.125
2.6 c. c.=.13
2.7 c. c.=.135
2.8 c. c.=.14
2.9 c. c.=.145
3 c. c.=.15

1+9=1:10 1 c. c.=.1
1.05 c. c.=.105
1.1 c. c.=.11
1.15 c. c.=.115
1.2 c. c.=.12

TABLES FOR DILUTING TOXINES FOR L+ DOSE—Continued.

1+9=1:10	1.25 c. c.=.125
	1.3 c. c.=.13
	1.35 c. c.=.135
	1.4 c. c.=.14
	1.45 c. c.=.145
	1.5 c. c.=.15
	1.55 c. c.=.155
	1.6 c. c.=.16
	1.65 c. c.=.165
	1.7 c. c.=.17
	1.75 c. c.=.175
	1.8 c. c.=.18
	1.85 c. c.=.185
	1.9 c. c.=.19
	1.95 c. c.=.195
	2 c. c.=.2
	2.05 c. c.=.205
	2.1 c. c.=.21
	2.15 c. c.=.215
	2.2 c. c.=.22
	2.25 c. c.=.225
	2.3 c. c.=.23
	2.35 c. c.=.235
	2.4 c. c.=.24
	2.45 c. c.=.245
	2.5 c. c.=.25
	2.55 c. c.=.255
	2.6 c. c.=.26
	2.65 c. c.=.265
	2.7 c. c.=.27
	2.75 c. c.=.275
	2.8 c. c.=.28
	2.85 c. c.=.285
	2.9 c. c.=.29
	2.95 c. c.=.295
	3 c. c.=.3

1+4=1:5	1 c. c.	=.2
	1.1 c. c.	=.22
	1.2 c. c.	=.24
	1.3 c. c.	=.26
	1.4 c. c.	=.28
	1.5 c. c.	=.3
	1.6 c. c.	=.32
	1.7 c. c.	=.34
	1.8 c. c.	=.36
	1.9 c. c.	=.38
	2 c. c.	=.4
	2.1 c. c.	=.42
	2.2 c. c.	=.44
	2.3 c. c.	=.46
	2.4 c. c.	=.48
	2.5 c. c.	=.5
	2.6 c. c.	=.52
	2.7 c. c.	=.54
	2.8 c. c.	=.56
	2.9 c. c.	=.58
	3 c. c.	=.6
1+1=1:2	1 c. c.	= .5
	1.1 c. c.	= .55
	1.2 c. c.	= .6
	1.3 c. c.	= .65
	1.4 c. c.	= .7
	1.5 c. c.	= .75
	1.6 c. c.	= .8
	1.7 c. c.	= .85
	1.8 c. c.	= .9
	1.9 c. c.	= .95
	2 c. c.	=1

THE ANTITOXIN.

Preparation.—The antitoxic serum was prepared at Parke, Davis & Co., Detroit, Mich., by Passed Asst. Surg. John F. Anderson, Assistant Director of the Hygienic Laboratory.

Two horses were selected which had furnished a reliable high potency serum. These horses had been treated in the usual manner by gradually increasing doses of the diphtheria toxine.

From horse No. 781 six liters of blood were drawn on June 14, 1904, at 9 a. m. The blood was drawn into 12 large test tubes, each tube containing approximately 500 c. c. Immediately after bleeding the blood was placed in a room, the temperature of which was about 25° C.

The next day, June 15, at 8.30 a. m., the horse was again bled for 6 liters, also into 12 tubes. This blood was placed with the lot drawn on the 14th.

After the blood had been clotted several days the clear serum was poured from the 24 tubes into a special bottle. Only that serum which was perfectly clear and entirely free from coloring matter was used. As the serum did not separate from this blood as clearly as usual, only 1,378 c. c. of serum were obtained from the 24 tubes. The serum was at once examined for potency and found to contain approximately 500 units. It was also tested bacteriologically for purity and no growths were obtained in bouillon tubes. After the samples were taken for testing, 20 c. c. of pure chloroform were put into the bottle with the serum, which was then at once placed in the ice chest at a temperature of 1.7° C. Chloroform was added to the serum to prevent any possible contamination. All of it of course disappeared with the vacuum under the drying process.

On June 22 evaporation of the serum was accomplished by bubbling dry air through the serum with a moderate vacuum. The vacuum varied from 20 to 23 inches. The arrangement for drying the serum is well shown in the accompanying illustration (fig. 7). The serum was syphoned into the large drying tube in small quantities, and the warm air bubbling through rapidly dried it and at the same time prevented the formation of hard cakes. The water bath surrounding the tube containing the serum was kept at about 35° C.; it varied from 32° to 36° C. throughout the operation. The temperature of the dry

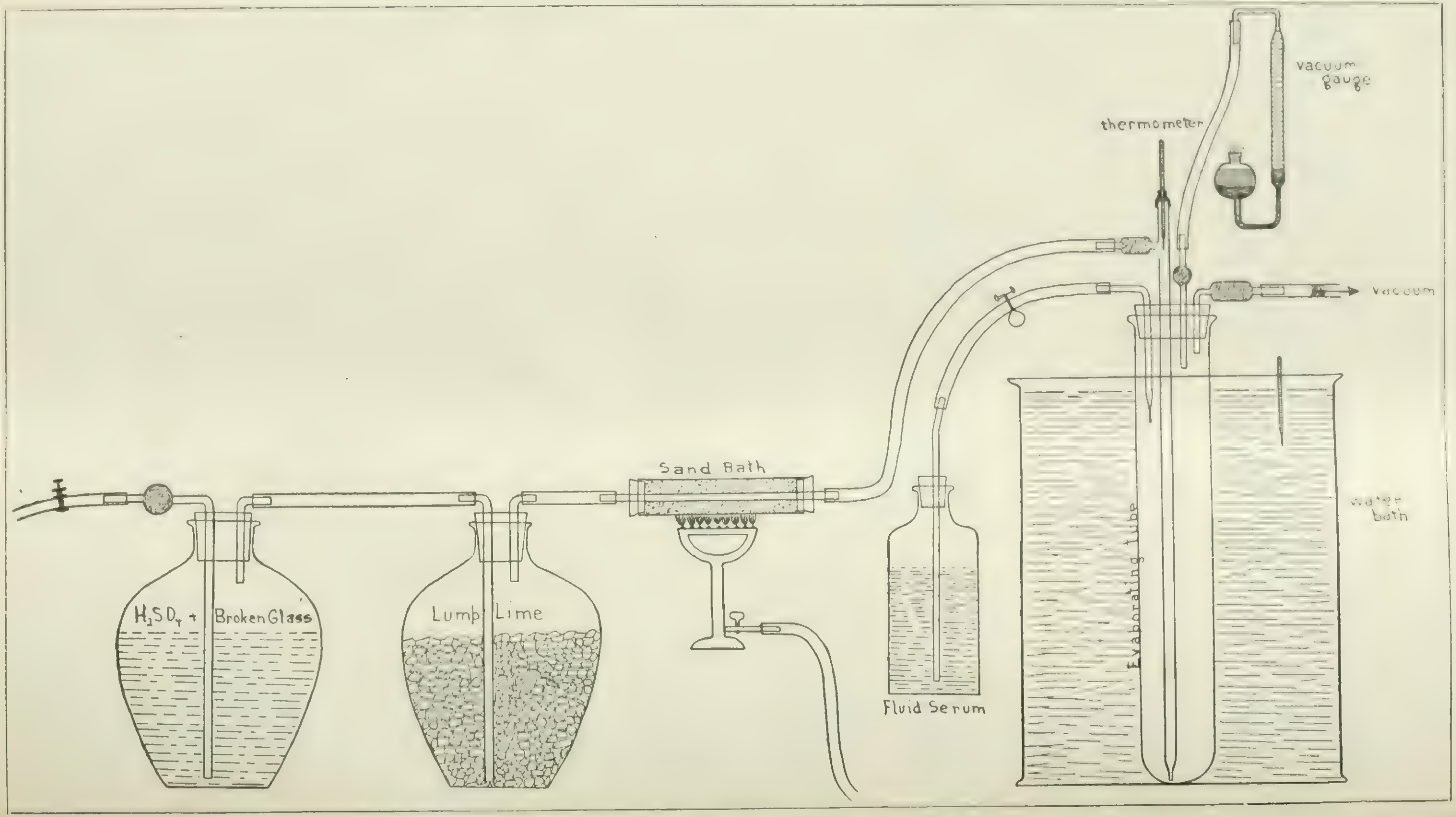


FIG. 7.—The apparatus used in evaporating the serum to dryness.

air passed through the serum was kept as nearly as possible at 33° C.; it never exceeded 35° C. The evaporation proceeded with fair rapidity. At the beginning there was some frothy bubbling, but that soon subsided. The entire lot of serum was dried in ten and a half hours, drying in large sugar-like flakes. Very little adhered to the side of the tube and there were no hard cakes.

The dried serum weighed 25 grams and was readily powdered in a sterile mortar to break up the larger lumps and then placed in a special porcelain grinder with porcelain balls and rotated for several hours. About half a gram of the powdered serum was planted in a tube of bouillon, which showed no growth at the end of four days.

Horse No. 590: This horse was also bled on two succeeding days—June 20 and 21—six liters being taken at each bleeding, as before. The 24 tubes, each containing half a liter of blood, were placed in a room with a temperature of approximately 25° C. After clotting two days the serum was poured off, only the perfectly clear fluid entirely free from coloring matter being retained. From the 12 liters of blood, 4,127 c. c. of serum were obtained. Samples were at once taken and tested for purity and potency, as before, with satisfactory results.

As soon as the samples were taken, 40 c. c. of pure chloroform were placed in the bottle with the serum, which was placed in the ice box at 1.7° C. Upon the same day evaporation of the serum was begun, proceeding as above described.

The evaporation of this amount of serum required 25 hours, the temperature of the air and water bath being the same as before. This serum dried in large golden-colored flakes, which were partly powdered in the sterile mortar and then put into the ball grinder and reduced to a fine, almost impalpable powder.

Four hundred and five grams of the dried serum were obtained. Half a gram was planted into bouillon, which showed no growth in three days, indicating that it had remained free from contamination throughout the operation.

Bottling and preserving the serum.—The dried serum must be kept guarded under special conditions to prevent deterioration. The oxygen of the air must be excluded in order to retard the process of oxidation, which rapidly alters the antibodies, especially under the influence of light. The serum is, therefore, always kept in absolute darkness.

The serum must be perfectly dry. It is therefore kept under the influence of phosphoric anhydrid, (P_2O_5), as shown in the accompanying illustration (fig. 8). Further, the serum is kept in a specially built ice box, maintained at a constant temperature of 5° C. by means of circulating brine.

The little bulbs in which the serum is preserved, shown in fig. 8, are made of amber glass. The following method is used in filling and sealing these bulbs:

Cotton is first introduced in the neck as far as the constriction. Then about 1 gram of phosphoric anhydrid is placed in one of the bulbs. The neck through which the acid has entered the little apparatus is now sealed off by the blowpipe. Then about 1.2 grams of the dried serum are placed in the other bulb. The neck, from the serum side, is connected with a vacuum apparatus, and as soon as the vacuum is sufficiently high this is also hermetically sealed.

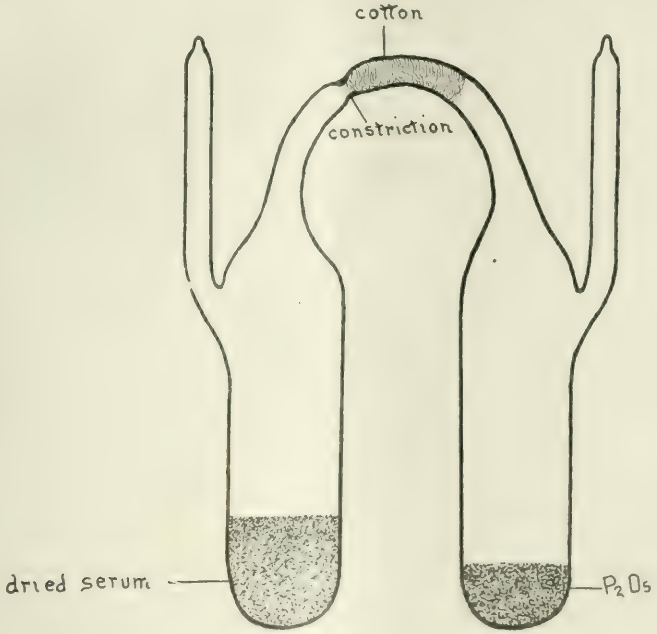


Fig. 8.—U shaped tubes used for preserving the standard serum. Exact size.

Each bulb is now sealed in a black box, which excludes the light, and is kept in the ice box at 5° C., as before stated.

From this arrangement it will be seen that the powdered serum is kept dry, free from contact with the oxygen of the air, guarded against the action of light, and preserved at a constant low temperature.

Dissolving the dried serum.—Once every two months, or whenever it is desired to test the serum, one of these bulbs is broken and 1 gram of the serum carefully weighed and dissolved in physiological salt solution (0.85) one part, Price's glycerin two parts.

Testing.—This glycerinated solution of blood serum is now accurately standardized in order to determine its precise antitoxic value in terms of Ehrlich's immunity unit.

The number of immunity units contained in a given quantity of the serum is determined by the test (L+) dose of toxine.

That quantity of serum necessary to add to the L+ dose of the toxine so that the mixture will kill the guinea pig on about the fourth day contains just one immunity unit. In the commercial testing of serums in order to have a coefficient of safety it would be safer to require the pig to live.

We use a number of toxines, the L+ doses of which have been very painstakingly ascertained on a large number of animals. The details of the technique by which this part of the testing is carried out will be found under the caption "Methods used in making the unit," and need not be repeated here.

THE METHODS USED IN MAKING THE UNIT.

The glassware.—The glassware employed in making the immunity unit for standardizing toxines and antitoxins is used for no other purpose. To a certain extent the glassware used for measuring and diluting the toxines is kept separate from that used for the serums.

The pipettes, burettes, graduated flasks, and mixing cylinders have been carefully calibrated or standardized by the Bureau of Standards, to whom we are under many obligations, not only for this work but for suggestions as to the most accurate methods of measuring precise amounts of fluids.

So far as possible all measurements are made with "capacity" instruments. "Outflow" instruments, made "to deliver" given quantities, are only used in the measurements of the final dilutions, in which case it is evidently impossible to use capacity pipettes.

The glassware used for the purposes of this special work has been calibrated with particularly fine graduations that pass halfway or all the way around the pipette, burette, or neck of the flask.

The diluting fluid is measured in mixing cylinders of a particular design made for me by Messrs. F. & M. Lautenschläger, of Berlin, Germany. Two of these mixing cylinders are shown in fig. 9. We have a series of these cylinders, each measuring quantities in intervals of 10 c. c. up to 100 c. c. The first set of cylinders is graduated to contain from 0 to 10 c. c., the next size from 10 to 20 c. c., then 20 to 30 c. c., 30 to 40 c. c., and finally 90 to 100 c. c. It will be noticed by the illustration that these mixing cylinders are subdivided into tenths, which makes it possible to measure our diluting fluid with great accuracy. It will also be seen that the cylinders have neither neck nor shoulder, and that the ground-glass stopper fits directly into the cylinder without a constriction. The internal diameter of these cylinders is just 12 mm., which readily permits the introduction of any one of our pipettes. The smaller pipettes average 6 to 8 mm. in outside diameter; the larger ones do not exceed 10 mm. Attention is also invited to the fact that the shape of the cylinders readily allows thorough mixing and cleansing.

We also use a style of mixing flask, shown in fig. 10, which was made for me by the Arthur H. Thomas Company, Philadelphia, Pa. The smaller flask is graduated to contain 49 and 50 c. c.; the larger flask is graduated to contain 98, 99, and 100 c. c. As we have frequent occasion to use these quantities, the flasks are particularly convenient on account of their simplicity and comparative cheapness. It will be noticed that the neck has no shoulder, or constriction.

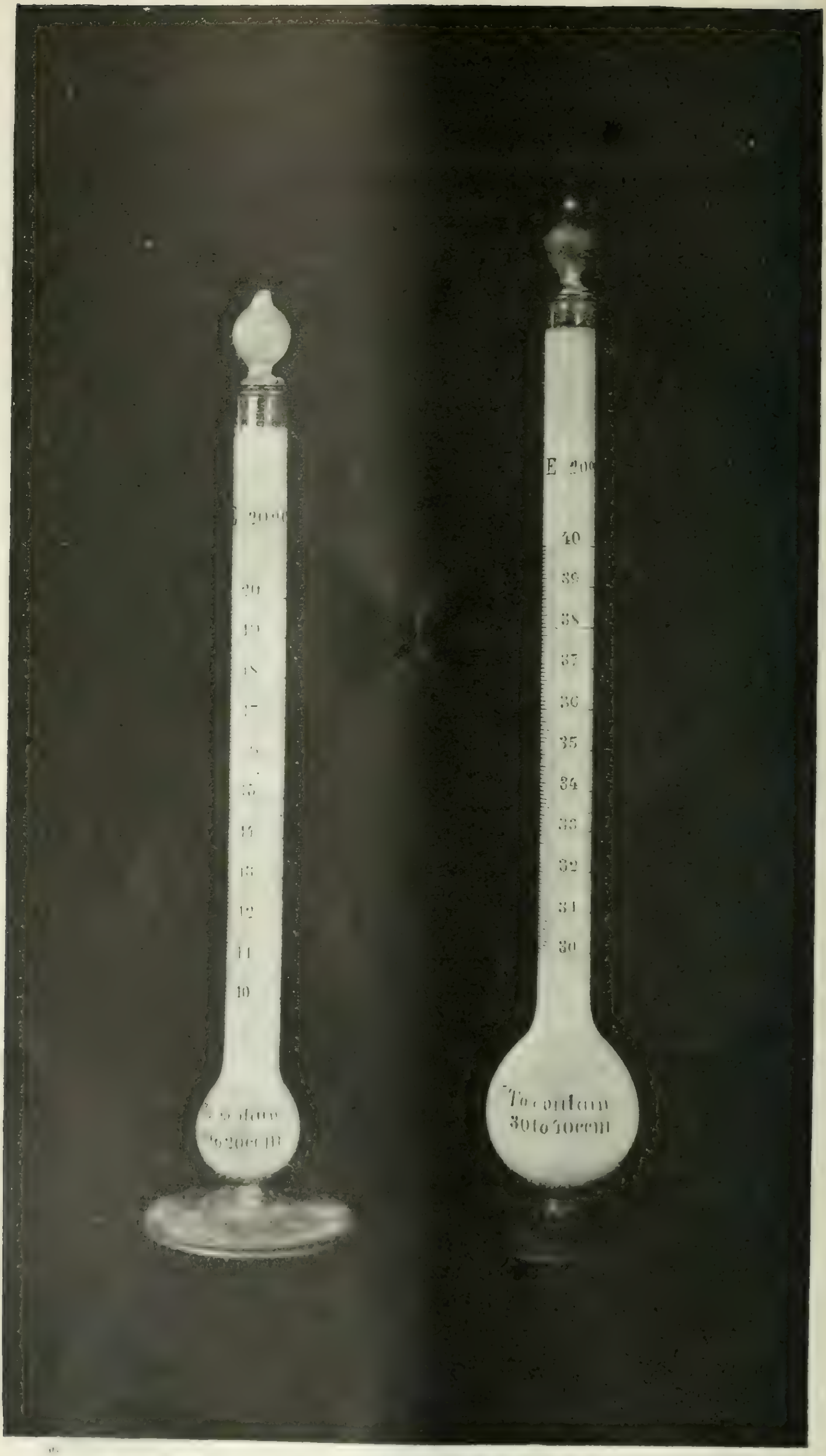


FIG. 9.—Special pattern mixing flasks for measuring toxine and serum dilutions.

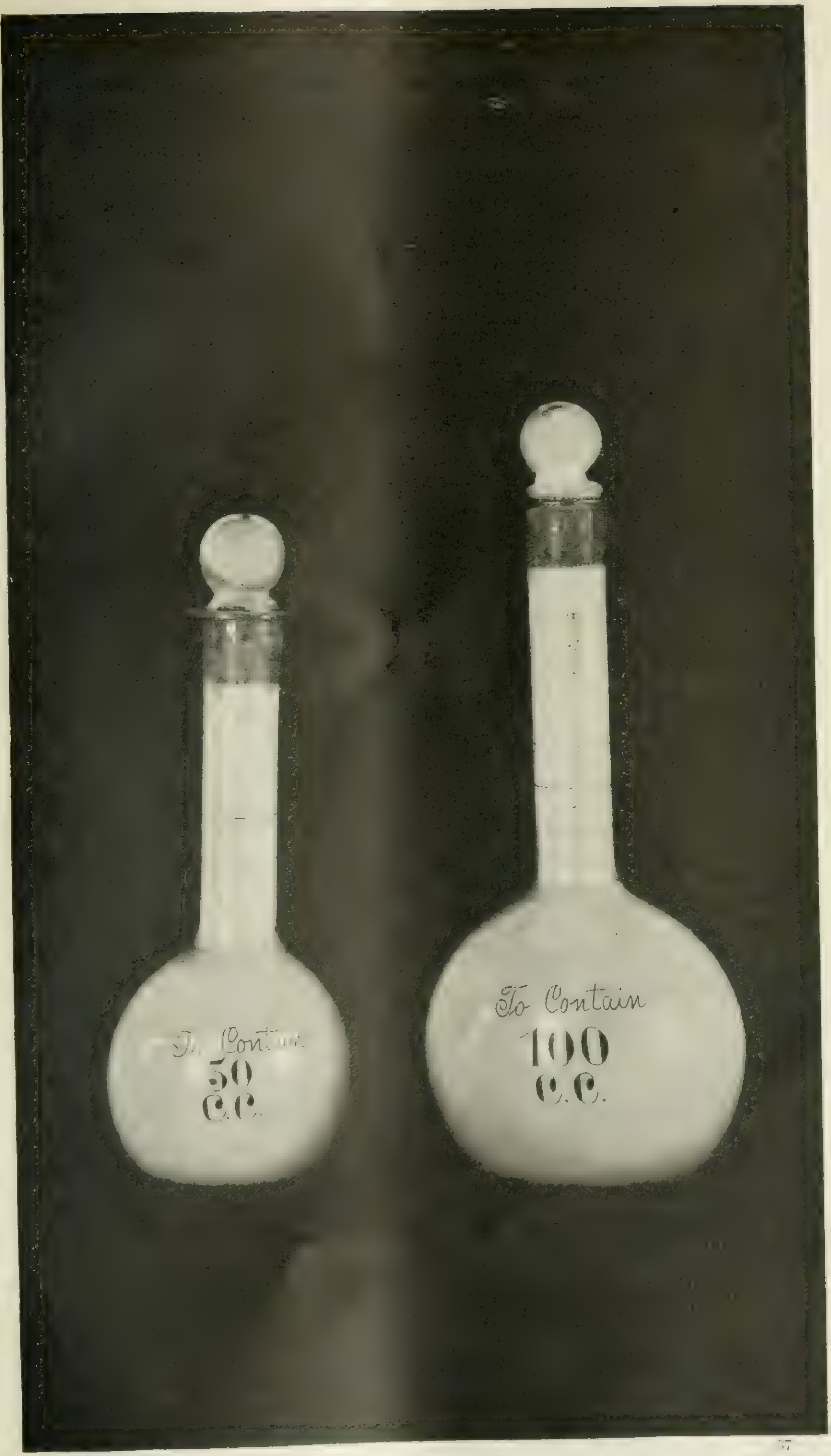


FIG. 10.—Capacity flasks for measuring dilutions of toxine, etc.

In reading the proper height of the fluid in these flasks the bottom of the meniscus is brought just to the line. By holding a black surface, such as a piece of cloth, beneath the level of the fluid the reflection makes the surface of the fluid stand out clearly as a single curved line, doing away with the annoying reflections that otherwise prevent accurate readings. This black curve of the meniscus is best seen against a white background.

The use of the "meniscus visir blende" of Doctor Göckel greatly facilitates the accurate reading of burettes, pipettes, and flasks. This little apparatus is exceedingly simple. It may be improvised in any laboratory, and will be found very useful.

All the glassware used in this work is cleaned by first flushing with cold, then with hot water, in order to remove the gross dirt. It is then bathed in a saturated solution of bichromate of potassium in concentrated sulphuric acid. The glassware is now flushed with tap water until all the acid is removed, when it is finally rinsed in several changes of distilled water and allowed to dry in the air.

All glassware is sterilized by heat at 125° C. for one hour, and kept under the usual precautions to prevent bacterial contaminations.

All the glassware used in this work has been standardized to contain or to deliver fluids at 20° C. instead of 15° C., because the former more nearly approaches the usual room temperature in this climate. The serums and toxines are removed from the ice chest a sufficient time before using to allow them to stand (in the dark) until they approach the room temperature before they are measured out.

The diluting fluid.—We use "physiological" salt solution (0.85 per cent. sodium chlorid C. P.) as our diluting fluid for all toxines, serums, etc. This salt solution is made up in bulk with distilled water and sterilized by steam. It is always used at room temperature

Bacteriological precautions.—All the work is done under the usual bacteriological precautions to prevent contamination. We do not observe the extreme precautions necessary to obtain a pure culture of an organism. The toxine and antitoxin are of course kept under perfectly sterile conditions and all the glassware with which they come into contact is clean and sterile, which appears sufficient to prevent contaminations entering the work that would affect the results. The autopsies made in this laboratory have so far failed to reveal any such infections of the guinea pig. Cultures were made from 154 consecutive guinea pigs. Some of the heart's blood was inoculated into bouillon tubes. Of these tubes 108 remained sterile; growth appeared in 46, as follows:

A coccus in	20
Motile bacillus in	18
Nonmotile bacillus in	5
Coccus and bacillus in	3
	46

A study of the histories of the guinea pigs showing the presence of bacteria in the blood disclosed the fact that most of them were probably either terminal or post-mortem infections.

METHOD OF USING THE PIPETTES.

In order to obtain accurate results with pipettes it is important to use them in the same way that they have been calibrated. This applies both to pipettes which have been graduated "to deliver" a given volume as well as to pipettes graduated "to contain" a given volume. If the method by which the pipettes were graduated can not readily be obtained from the firm making them, it will be necessary to restandarize them to determine several points.

With delivery pipettes it is essential to know whether they are to be used by blowing out the last drop or by touching the point of the pipette to the surface in order to remove the last drop that adheres to the tip.

The time allowed for drainage, after the pipette is emptied, varies with the rate of flow and also with the standard methods used by different countries.

It is furthermore very important in accurate work to know whether pipettes have been graduated in accordance with the Mohr liter or the true (French) liter.

The Mohr liter is the volume occupied by 1 kilogram of pure water at 17.5° C., weighed in air against brass weights, the air being under normal pressure and at 17.5° C. The volume of such a liter is 1,002.36 c. c.

The true or so-called French liter is the volume occupied by 1 kilogram of pure water at 4° C., the weighing being reduced to a vacuum. The volume of the true liter may be assumed equal to 1,000 c. c.

Volumetric apparatus is for convenience usually graduated to contain or to deliver true cubic centimeters at higher temperatures than 4° C., namely, at 15°, 17.5°, or 20° C., the standard temperature being marked upon the apparatus. The differences between apparatus graduated on the true and on the Mohr systems are as follows:[a]

If standard at 15°, Mohr apparatus is larger by 2.30 c. c. per liter.
If standard at 17.5°, Mohr apparatus is larger by 2.36 c. c. per liter.
If standard at 20°, Mohr apparatus is larger by 2.42 c. c. per liter.

In ordinary rough-and-ready work all these refinements may be disregarded, but in certain determinations requiring great precision a neglect of these details may cause serious error.

These matters were brought forcibly to my attention in the work of making the standard unit for diphtheria antitoxin.

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[a] I am indebted to Dr. L. A. Fischer, Chief of the Division of Weights and Measures, Bureau of Standards, for these figures.

The errors in using pipettes may be especially manifest in measuring solutions of higher density or greater viscosity than water. In fact it is quite impossible to obtain even approximately correct results in measuring such solutions as glycerin, blood serum, etc., from delivery pipettes. This fact led Ehrlich to use the capacity pipettes of von Leybold in measuring these substances in making his unit for diphtheria antitoxin.

I have found the methods which I shall now describe very useful in the accurate work necessary in making a Government standard and believe their simplicity and accuracy will commend them to others.

Method of using capacity pipettes.—The pipettes are fitted with a rubber bulb at their upper end and held in an ordinary retort stand, as may be seen by reference to the illustration (fig. 12).

The rubber bulb is clamped between the jaws of the compressor. The fluid is drawn up into or expelled from the pipette by tightening or loosening the screw. By this means we have an absolute control over the height of the column of fluid in the pipette. The rubber bulb takes the place of the suction usually applied by the mouth and is used both to fill and to empty the pipette.

In using, the free end of the pipette is immersed into the fluid to be measured, and by loosening the screw the column of fluid is gradually brought approximately to the mark. The operation is repeated once or twice so as to wash the pipette with the fluid to be measured, which is finally drawn a little above the graduation mark. Care must be taken not to wet the interior of the pipette at any time during the process of measuring more than 1 to 2 mm. above this mark, because the extra wetted surface would furnish an appreciable error. By holding the pipette in the retort stand and using the rubber bulb as shown, such accidents are much less likely to happen than is the case when the mouth is used to suck the fluid up and down.

The outside of the pipette is now carefully wiped with a few thicknesses of sterile gauze. Then by placing the gauze to the free end of the pipette the column of liquid may be slowly drawn down until the meniscus rests directly upon the line.

The contents of the pipette are now emptied into the diluting fluid, which has previously been measured out, and the pipette must be washed out thoroughly by drawing the fluid up and down several times, again using the rubber bulb for this purpose.

The flask containing the dilution is now agitated so as to obtain a uniform mixture of solution, and the pipette is again washed in and out so that the fluid which adheres to and remains in the pipette is the same dilution as the mixture in the flask. A larger quantity of dilution is always made than required, so that aliquot parts will represent very definite quantities of the original substances.

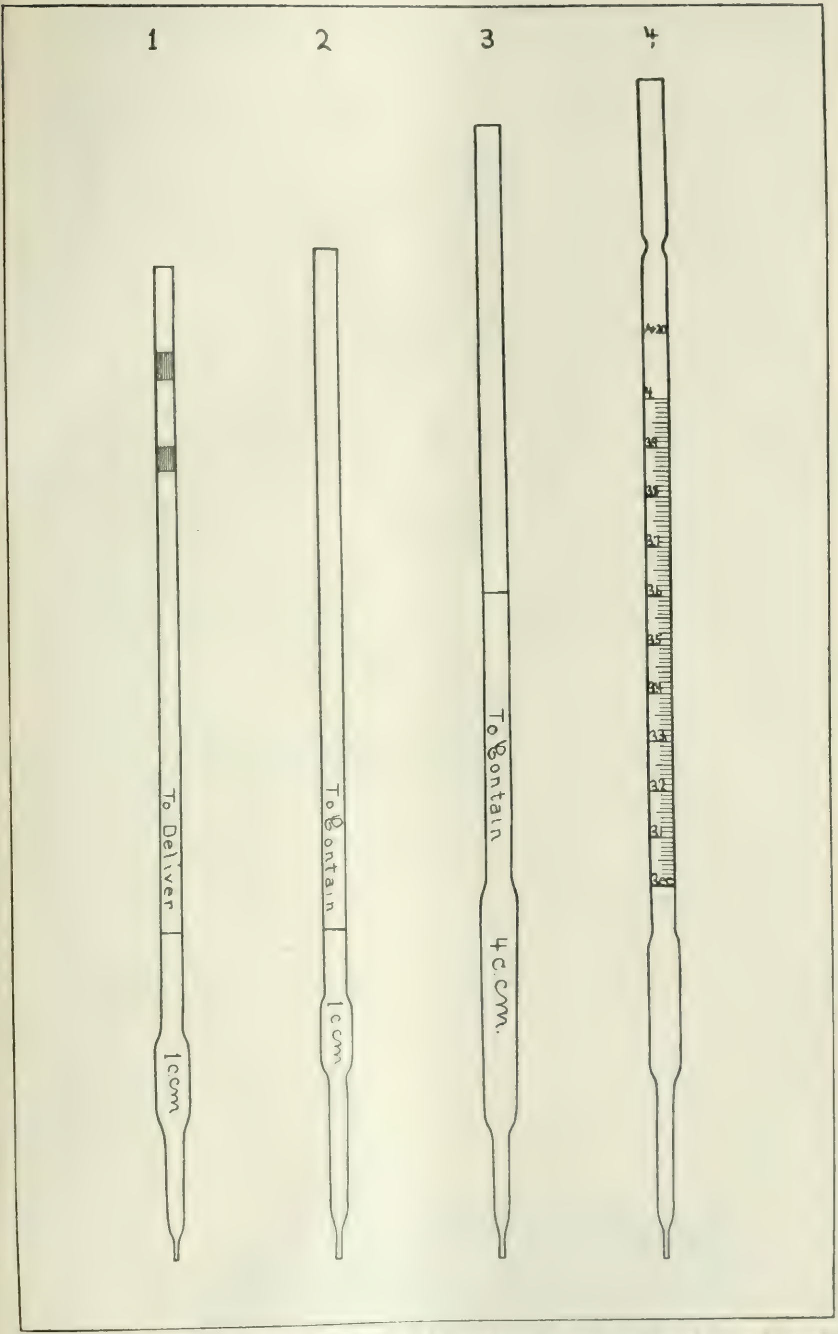

Fig. 11.—Various pipettes used in the work. 1. A delivery pipette, with two etched bands for ready identification, graduated to blow out the last drop. 2. A 1 c. c. capacity pipette. 3. A 4 c. c. capacity pipette. 4. An Ehrlich delivery pipette graduated in hundredths.

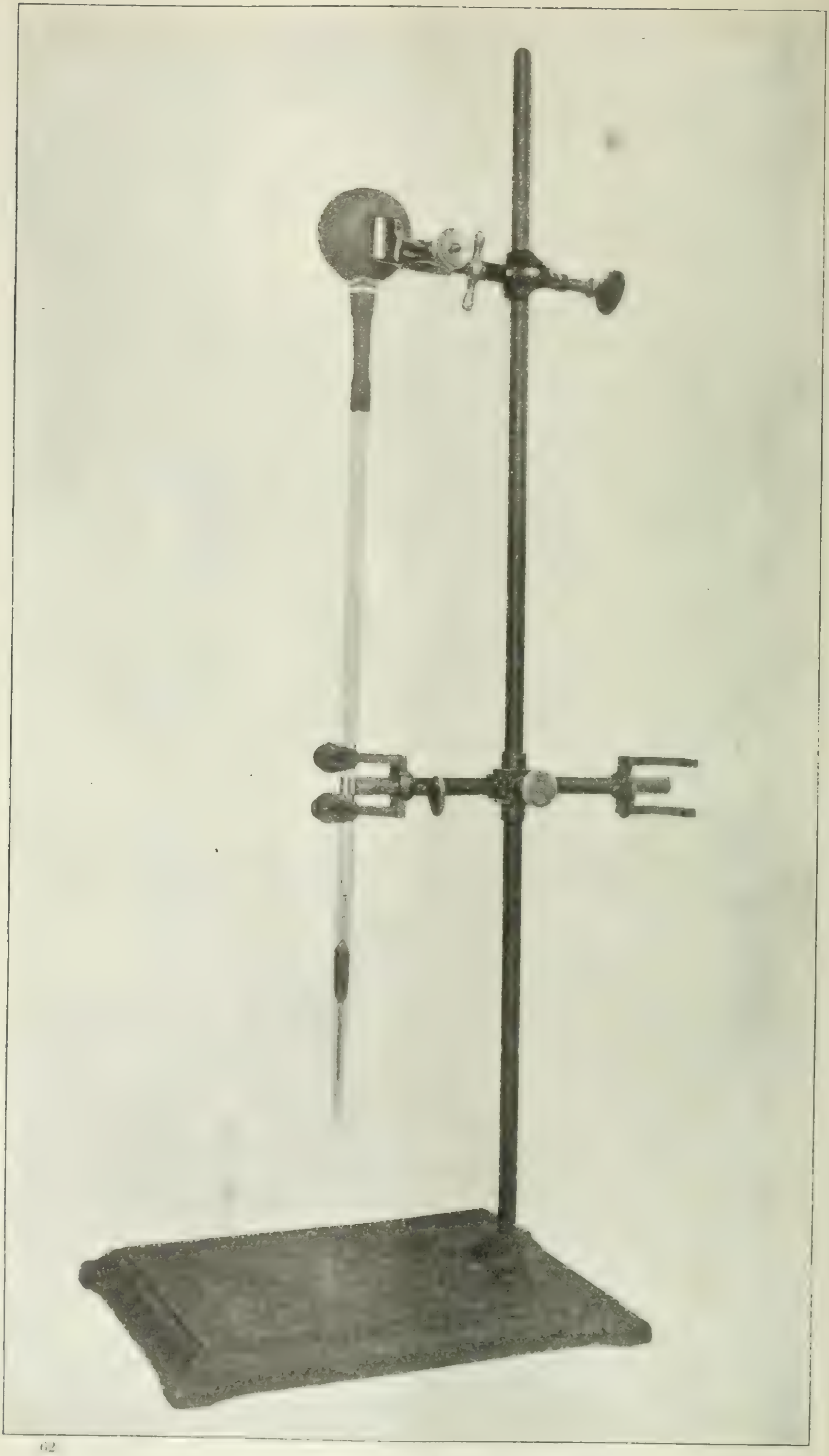

FIG. 12.—Method of using capacity pipettes.

The whole process does not take as long as the telling, and is much quicker and more accurate than the ordinary method of using the pipettes with a free hand.

Method of using delivery pipettes. The method of using "outflow" or "delivery" pipettes differs materially from that of using capacity pipettes.

The rubber bulb is attached to the pipette as before, but in this case there is a T between the bulb and the pipette. From this T a small piece of rubber tubing, controlled by a pinchcock, is located, as may be seen by reference to fig. 13.

The pinchcock is kept closed while the pipette is filled to the proper graduation, which is accomplished just as in the case of the capacity pipettes described above.

By opening the pinchcock the contents of the pipette are allowed to flow out. When the last drop that will flow out of its own accord escapes, the proper time is allowed to elapse for drainage and the point of the pipette touched to the surface. By this procedure a very constant quantity of fluid remains in the capillary tip of the pipette.

If the pipettes have been standardized to blow out this last drop, this may be done conveniently by closing the pinchcock and compressing the rubber bulb.

The soft rubber ear syringes sold at any drug store make very good bulbs for this work. It is only necessary to cut off a small portion of the nozzle of the ear syringe. The thicker-walled rubber bulbs made for Koch syringes respond more quickly and are more durable. The illustrations show the rubber bulbs attached directly to the mouthpieces of the pipettes. We have found it more convenient to bring the bulb by means of glass and rubber tubing to the base of the retort stand.

I have used pipettes in accordance with these two methods during the past twelve months in work with toxines and antitoxins, and have concluded that they are quicker, more accurate, and more satisfactory than the old freehand technique.[a]

In working out the problems connected with the accurate methods of measuring given volumes of toxine and antitoxin, I have had the help of the Bureau of Standards, and am indebted especially to Dr. L. A. Fischer and Mr. N. S. Osborne, of the Bureau, for many useful suggestions.

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[a] This method was demonstrated at a meeting of the Society of American Bacteriologists, Philadelphia, December 27, 1904.

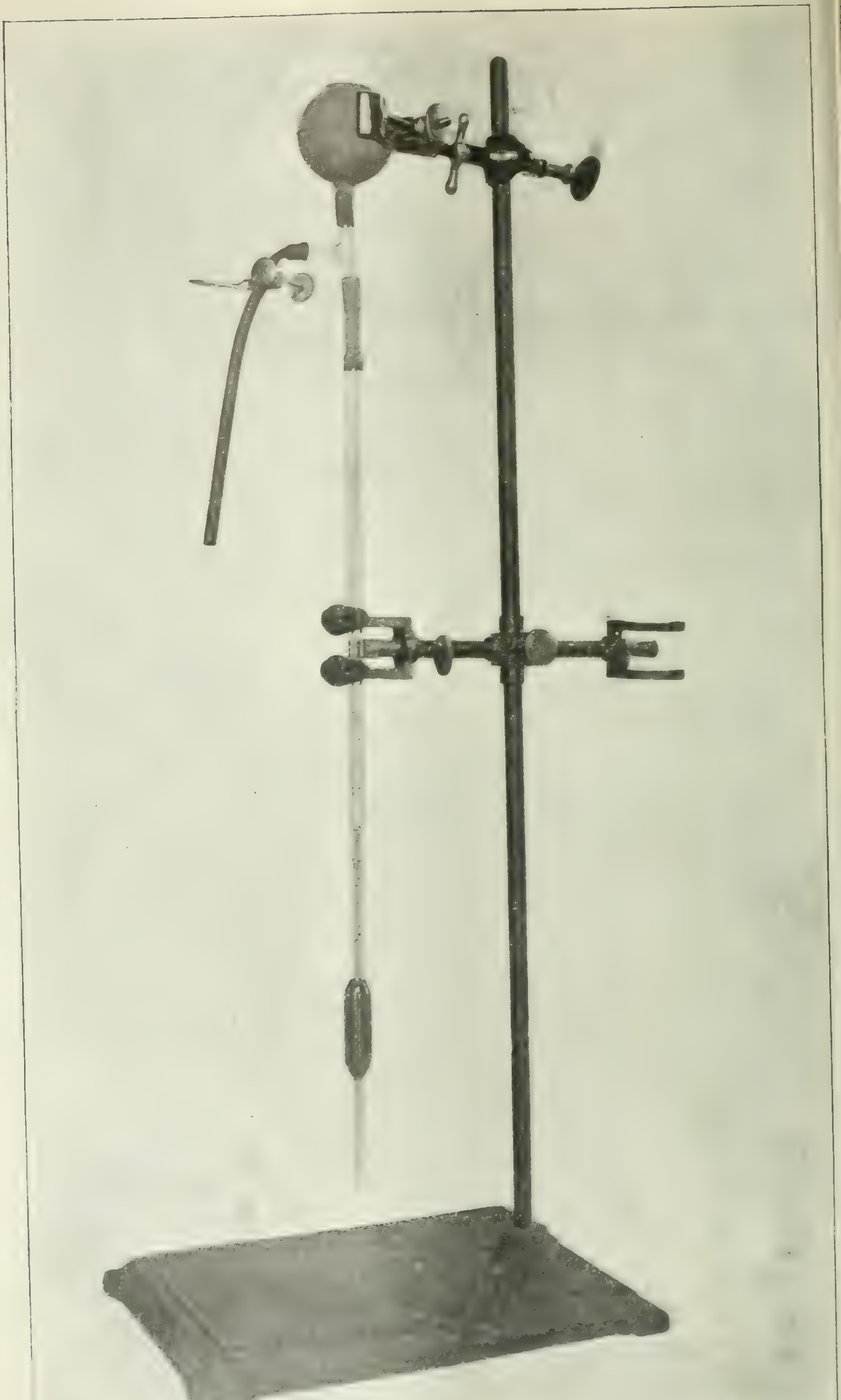

FIG. 1[illegible].—Method of using delivery pipettes.

METHOD OF INOCULATING THE ANIMALS.

The method used for inoculating the animals has been described in Bulletin No. 19 of the Hygienic Laboratory, Public Health and Marine-Hospital Service, entitled "A method for inoculating animals with precise amounts," and only the essential features as applicable to this work will be repeated.

The syringe used for carrying out this technique is a modification of the old Koch syringe.

The needles are sterilized separately by the usual method of boiling in a 1 per cent sodium carbonate solution.

The needle is then screwed on the barrel of the syringe and the joint tested by drawing some sterile salt solution in and out several times. If the joint is tight and the needle pervious, the outside is dried with a little piece of sterile gauze and the needle is now plunged into a jar of sterilized albolene. The albolene acts as a temporary plug, preventing any of the fluid that is placed in the syringe from escaping until it is injected into the animal.

The necessary number of syringes are prepared, one for each animal. In testing diphtheria toxine and antitoxin we sometimes have a battery of fifty or sixty thus prepared, arranged on the rack as shown in the accompanying diagram (fig. 15).

The amount of fluid desired to inject into the animal is now carefully measured directly into the barrel of the syringe. This is injected into the animal and then, without withdrawing the needle, the bulb is removed. The necessary quantity of salt solution is quickly run into the barrel, washing down the sides, and this in turn is injected into the animal.

When the syringes contain fluid they can not be inverted, as is the case with a piston syringe. It is not necessary, how-

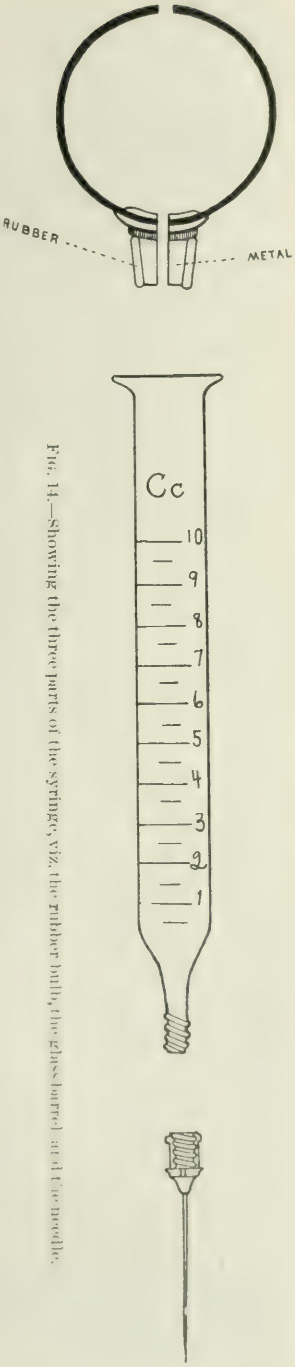

Fig. 14.—Showing the three parts of the syringe, viz, the rubber bulb, the glass barrel and the needle.

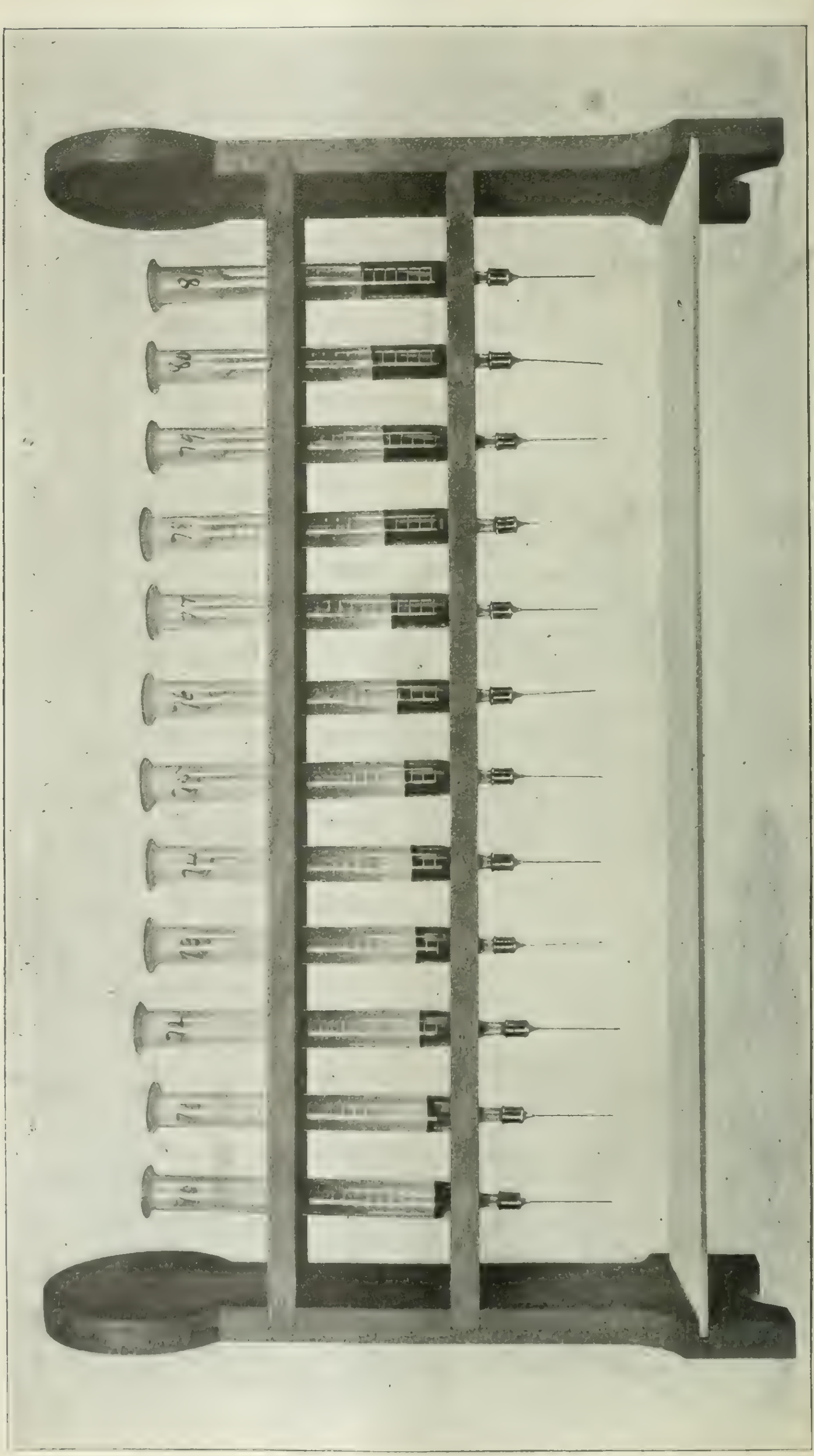

FIG. 15.—A battery of 12 syringe barrels ready for use. The bottom shelf is of milk-white ground glass to show a leak and is used to write upon.

ever, to hold them upright, as might at first be supposed. They may be tilted to a greater angle than 45° without danger of the fluid running out.

Each glass barrel is numbered with the number of the experiment or of the animal to receive the injection. This is convenient and avoids confusion.

The syringes are prepared and filled in a special room where the exact measuring is done, and the rack is then found handy to carry the syringes to the animal room where the work of injection is carried on.

In using this method in standardizing diphtheria antitoxin it is very important to shake and roll the syringe very actively so as to obtain an intimate mixture of the toxine with the serum.

The toxine is first measured into the syringe barrel, then the diluted serum. The two are thoroughly mixed by agitating the syringe and the mixture allowed to stand at room temperature in diffused light one hour before it is inoculated into the guinea pig.

The dilutions are so arranged that the guinea pig always receives a total of 4 c. c. of fluid, thereby insuring constant pressure effects.

We always dilute our serums so that the amount of antitoxin desired will be contained in just 1 c. c. of the dilution. It is preferable to dilute the toxines so that the amount of desired poison will be contained in 2 c. c. or less of solution. In this way we have at least 1 c. c. left to make up the total of 4 c. c. with which to wash out the syringe barrel.

THE GUINEA PIG.

In this work much depends upon the guinea pig. Animals sometimes show such marked idiosyncracies in their reaction to certain poisons that it is remarkable in case of diphtheria toxine that a guinea pig should show comparatively little of this individuality. It may be stated in general terms that the guinea pig as a vital factor in the standardization of diphtheria antitoxin is quite as dependable as our ordinary weights and measures.

It is of course necessary to use pigs under standard or normal conditions, of a definite weight, and approximately the same age. They should be bred from reliable stock, and fed and caged so as to insure vigorous animals.

In order to meet all the exacting requirements we raise our own guinea pigs, thus insuring animals of reliable and constant conditions. Our pigs are fed upon oats and green food such as cabbage, carrots, and grass in season.

Theobald Smith[a] has recently shown that some female guinea pigs give birth to progeny all of which seem to show a marked resistance to the diphtheria toxine. Such hereditary influences, which would seriously affect the results, should be guarded against by elimination of this stock.

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[a] Smith, Theobald: Degrees of susceptibility to diphtheria toxin among guinea pigs. Transmission from parent to offspring. Jour. med. res., v. 13 (3), p. 341.

Weight of the guinea pig.—Ehrlich prescribes the use of guinea pigs weighing 250 grams. We consider a pig weighing anywhere from 250 to 280 grams as complying with this requirement.

The weight of a young guinea pig varies with the quantity of food and water taken and the activity of metabolism. We have found young hungry pigs to eat as much as 35 grams in a day. The weight of a young pig will ordinarily change from 10 to 20 grams in a day, depending upon the food and water taken. We, therefore, always weigh our guinea pigs in the morning before feeding time. We sometimes use guinea pigs weighing more than 280 grams, but less than 350 grams, for our first work on a toxine or serum to determine its approximate strength.

In our experience a guinea pig weighs about 100 grams at birth, and is between six and eight weeks old before it weighs the required 250 grams. Occasionally, when the mother gives birth to only one, we have found, under such favorable conditions, that this single offspring may gain so rapidly as to weigh 250 grams before it is four weeks old.

We do not always have enough guinea pigs of our own raising to carry on the work. In that case we buy from reliable dealers, insisting on pigs weighing less than 225 grams, so that we never use a pig that has not been in the laboratory under observation ten days or two weeks.

The pigs are weighed upon a spring-balance scales. These scales are sufficiently accurate, weighing to 2.5 grams, and are much quicker and more convenient than pan balances with weights.

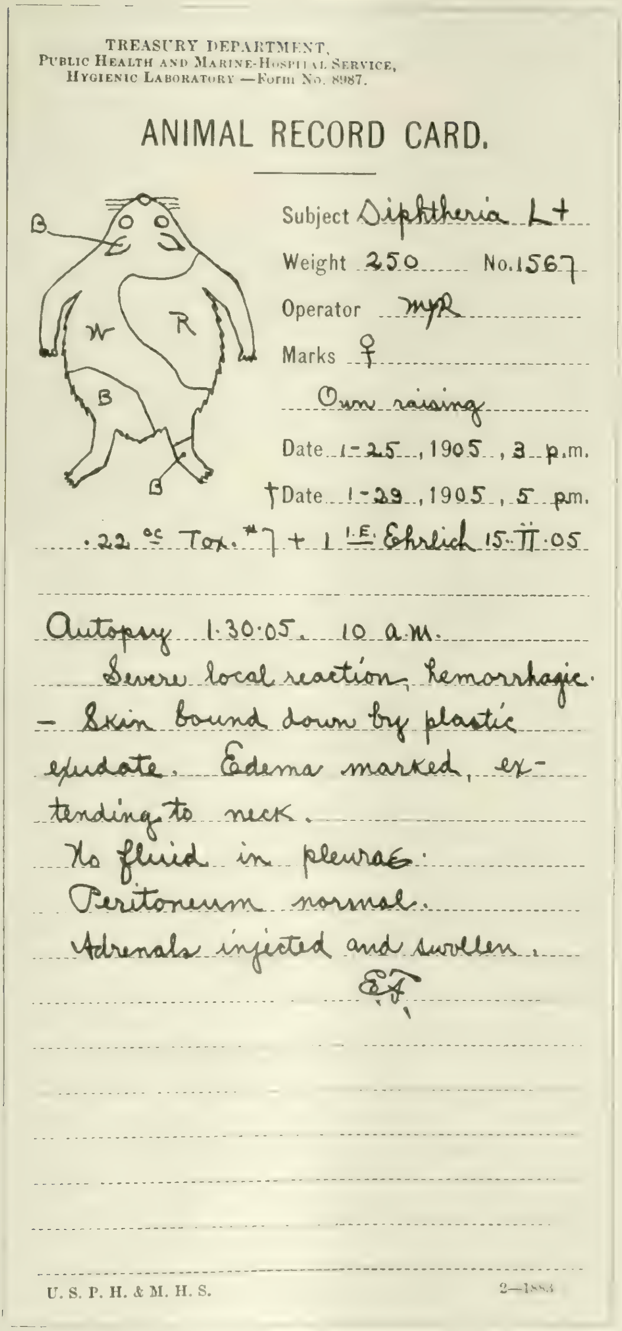
As soon as the young pigs are old enough to be weaned they are divided, separating the males from the females. Pregnant female pigs should not be used in this work.

A pig having been selected and weighed has a card made for it with a rough diagram, on which is indicated its peculiarities of color or other marks. This is a satisfactory method of identifying the animal. The character of card is here shown (fig. 16).

Technique of operation. The abdomen is shaved the day before operation, clearing the site of operation so that we may readily see just what we are doing. An area about 2 inches in diameter is shaved.

The preparation of the animal card shown in fig. 16, the shaving, etc., is done the day before the operation, so that the pig is not handled more than necessary to receive the injection on the day of the operation.

The pig is held by an assistant and the needle introduced in the left flank from one-half to an inch above the supraspinous process of the



TREASURY DEPARTMENT,
Public Health and Marine-Hospital Service,
Hygienic Laboratory —Form No. 8987.

ANIMAL RECORD CARD.

Subject Diphtheria L+
Weight 250 No. 1567
Operator MJR
Marks ♀
Own raising
Date 1-25, 1905, 3 p.m.
†Date 1-29, 1905, 5 p.m.
.22 cc Tox. #7 + 1 I.E. Ehrlich 15·II·05

Autopsy 1·30·05. 10 a.m.
Severe local reaction, hemorrhagic. – Skin bound down by plastic exudate. Edema marked, extending to neck.
No fluid in pleurae.
Peritoneum normal.
Adrenals injected and swollen.
E.F.

U. S. P. H. & M. H. S. 2—1883

Fig. 16.—Animal record card.

ileum. The needle is introduced its whole length (1.25 inch) strictly subcutaneously toward the middle line. This part of the operation is arranged so that the point of the needle will be just about at the linea alba.

It is of the greatest importance to keep the needle out of the peritoneal cavity, and care must also be exercised not to pierce the skin too superficially, and it is essential to keep out of the muscles.

Probably no part of the entire procedure is more important than the inoculation of the toxine and antitoxin into the proper tissue of the guinea pig. By always injecting the toxine into the same tissues we insure three factors: (1) The rate of absorption, (2) the pressure effects are relatively the same in the same anatomical structures, and (3) the relation between the toxine and the receptors of the cells and body juices is comparatively constant when the injection is made into the same anatomical structures. If any of the toxine enters the peritoneal cavity the rapid absorption may result in the untimely death of the pig with an extensive hemorrhagic reaction.

Still greater care must be exercised to keep the toxine out of the muscles. This point was brought out by Ehrlich and has since been emphasized by S. J. Meltzer[a] and John Auer in their work "On the rate of absorption from intramuscular tissue," with curare and adrenalin.

It has been shown that the rate of absorption from muscular tissue is so active that poisons injected into the muscles are apparently two and three times as strong as when injected into the subcutaneous tissue.

Ehrlich selects for the site of injection the skin at the median line in the region of the xiphoid process of the sternum, and is careful to insert the needle purely subcutaneously by pinching up the skin which is loose in this region.

For the purposes of determining at autopsy the exact site at which the toxine and antitoxin mixtures have been inoculated, little particles of burnt cork are used by Ehrlich. In this laboratory we use a little sterilized powdered animal charcoal, simply for its convenience. Upon dissection the particles of carbon may plainly be seen and show the place where the fluid was injected. This technique has its particlar value in determining the L^0 dose.

We have found that if the fluid enters the proper abdominal layer it produces an egg-shaped swelling parallel to and overlying the linea alba.

As soon as the toxine-antitoxin mixture is injected into the animal the rubber bulb is removed and an assistant adds the necessary amount of salt solution to make up the 4 c. c. which, after washing down the sides of the syringe, is in turn forced under the skin. Care to avoid the injection of an unnecessary amount of air should be taken.

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[a] Meltzer & Auer: On the rate of absorption from intramuscular tissue. Am. med. (abstract), v. 9 (102), Jan. 14, 1905, p. 75.

The inoculation should be done rapidly and without unnecessary violence, which may affect the results. A trained assistant will hold the guinea pig, without causing struggling, and an experienced operator will cause little pain other than the prick of the needle through the skin.

Care must of course be taken in inoculation that none of the fluid escapes. We do not attempt to disinfect the skin.

Effect upon the guinea pig.—After inoculation the guinea pigs are kept two in a cage and not disturbed until the seventh day. Keeping many animals together in a pen may give irregular results, because the strong and lusty pigs worry the sick ones and may hasten their end. Examining the pigs too often or too roughly must also be avoided. Theobald Smith believes that palpating the edema, caused by the reaction of the toxine at the site of inoculation, may cause the poison to break beyond the confines nature is setting up as a barrier, and may thus hasten the result. We have found that there is little to be learned in weighing or examining pigs during the first week, and we therefore leave them undisturbed during this time. They are weighed on the seventh day and each week following until the fourth week, when, if gaining weight and in good condition, they are passed from observation.

Guinea pigs that show late effects of *toxone*, as indicated by paralysis, will do so some time after the fourteenth day and before the thirtieth day with great regularity. The facts of paralysis, sloughs, ulcers, etc., are carefully noted on the records.

When an animal dies an autopsy is always made in order to insure that the lesions are typical, and to guard against the possibility of infections—pneumonia, tuberculosis, pseudotuberculosis, etc., and to insure the fact that the toxine was not injected into the muscles or the peritoneum—which may have rendered the animals more susceptible.

The guinea pig shows a remarkable constancy in its reaction to varying amounts of the diphtheria poison. Overpowering doses of the toxine—say, 100×MLD almost invariably kill the animals in about twenty-four hours. It is with the rarest exception that a guinea pig will die as a result of the diphtheria poison in less than twenty hours. Smaller amounts cause the death of the animal in from thirty-six to forty-eight hours. As we approach the limit of the minimal lethal dose, or the mixture containing the L+dose of the toxine and one immunity unit, we find one of three results:

(1) The animal dies from acute poisoning on about the fourth day.

(2) The animal develops post diphtheritic paralysis between the fourteenth and thirtieth days.

(3) Recovery.

The first result, viz, the acute death of the guinea pig, is of special interest to us, because it is the factor which determines the strength of the toxine and antitoxin.

Guinea pigs dying acutely do so with great regularity on the fourth day, the actual time averaging about three days twelve hours. Occasionally guinea pigs will die between two and a half and three and a half days as a result of the injection of a minimal lethal dose or as the result of an injection of the mixture of toxine and antitoxin containing the L+ dose of the former and one immunity unit of the latter. In these cases the autopsy will often show a reason for this lack of resistance indicated by the severity of the reaction and especially the presence of pleuritic exudates, or the guinea pig may have pneumonia or some other disease. Occasionally more resistant animals will die on the fifth day, rarely on the sixth day, and very exceptionally on the seventh, eighth, or ninth days.

If the poison is not sufficient to kill the animal acutely as above described, paralysis may supervene. The symptoms of paralysis do not appear before the fourteenth day and usually show themselves in the legs. The hind legs are commonly affected first. The guinea pig not unusually recovers after marked paralytic symptoms, but ordinarily the fore legs become limp and useless, respiration indicating phrenic involvement makes its appearance, and death supervenes.

EXAMINATION OF SERUMS MADE BY LICENSED MANUFACTURERS.

The act of Congress approved July 1, 1902, entitled "An act to regulate the sale of viruses, serums, toxins, and analogous products in the District of Columbia, to regulate interstate commerce in said articles, and for other purposes," and the regulations framed thereunder, approved February 21, 1903, imposed upon the Director of the Hygienic Laboratory the duty of examining vaccines and antitoxins for purity and potency.

Accordingly purchases are made for the Hygienic Laboratory from time to time on the open market by officers of the Public Health and Marine-Hospital Service stationed in various parts of the country. The antitoxin is always bought from reliable druggists who keep the product under proper conditions of light, temperature, etc. Several grades of diphtheria antitoxin made by each licensed manufacturer are bought and sent to the Hygienic Laboratory by mail for the purposes of these tests.

The serums are tested not only for potency, but also to determine their freedom from contamination by foreign bacteria, and finally to insure the absence of chemical poisons, especially tetanus toxin. Note is made of the physical appearance of the serum, and tests are made to determine whether an excessive amount of preservative has been added.

A careful memorandum is made of the facts given by the manufacturer, as stated upon the label, as to the number of units contained in the package, and the date beyond which the contents can not be expected beyond a reasonable doubt to yield a specific result. Note is also made of the manufacturer's compliance with the law requiring that the product be plainly marked with the name of the article, and the name, address, and license number of the manufacturer.

Delinquencies that occasionally come to light in these examinations are at once reported to the Surgeon-General, U. S. Public Health and Marine-Hospital Service, who takes the necessary steps requiring the immediate withdrawal of the particular lot of serum from the market and institutes measures to prevent a repetition of similar errors.

In examining the unit strength of antitoxin sold by licensed manufactures the first step is to measure carefully the quantity of fluid contained in the syringe, bottle, or special container, whatever it may be. For this purpose the serum is emptied into a cylinder that has been standardized by the Bureau of Standards in which the total amount of the serum may be accurately read to the fractional part of a cubic centimeter.

From 0.5 to 1 c. c. of each lot of serum is planted into a large amount of bouillon in order to dilute the preservative. This is incubated at body temperature and any growth that may appear is determined by the usual bacteriological methods.

The number of units that the serum contains is then estimated by the following procedure:

If a syringe contains 4 c. c. of serum and is labeled 1,000 units it must of course have at least 250 units to each cubic centimeter. We would then test such a sample upon four or five guinea pigs to see whether its unit strength is above or below the value claimed for it. If the strength of the serum is 10 per cent below that claimed, a special report is made to the Surgeon-General to this effect, the manufacturer is notified that all the serum bearing this laboratory number must be withdrawn from the market, and the matter is investigated.

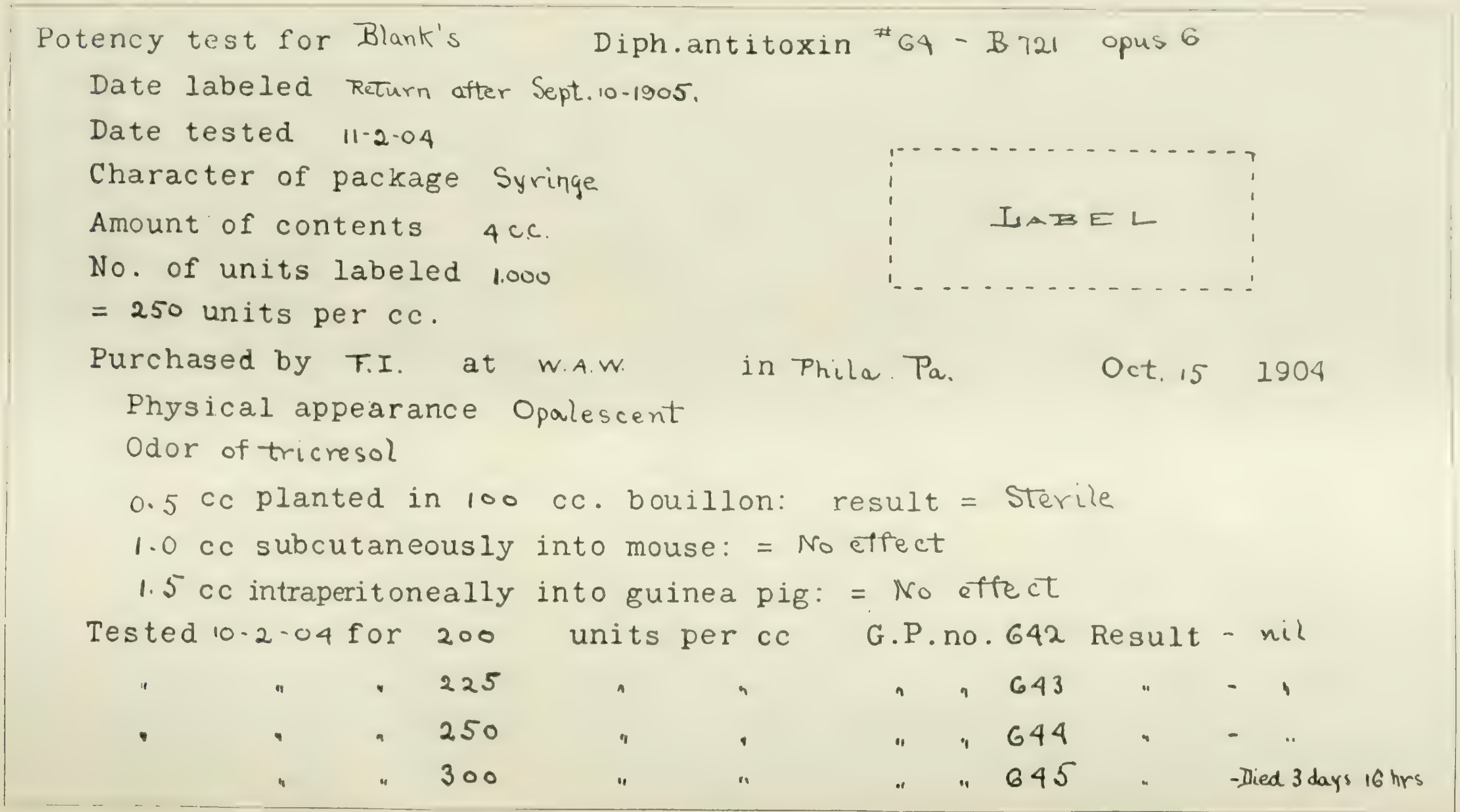
The tests to determine the strength of commercial serums sold upon the open market are made against a toxine that has been very carefully tested to determine its exact L+ dose. Controls at the same time are always made against both Ehrlich's normal serum and the official standard serum made in this laboratory. In case of doubt or contradictory results the work is always repeated, making the second tests against the L+ doses of two toxines.

As weak serums are of doubtful therapeutic value, we do not favor the sale of diphtheria antitoxic serum containing less than 250 units per c. c. If a serum bought on the market contains less than 200 units per c. c., the fact is reported to the Surgeon-General, who requests its withdrawal from sale and a discontinuance of the practice.

To determine that an excessive amount of preservative belonging to the phenol group has not been added, the following tests, modified from similar tests suggested by Ehrlich, are employed:

It has been found that 0.5 c. c. of a 0.5 per cent solution of phenol will kill a medium-sized mouse if injected subcutaneously. The quantity of phenol contained in 0.5 c. c. of such a solution represents 0.0025 gram. It requires 1 c. c. of a 0.5 per cent solution of tricresol to produce the same result. One c. c. of a 0.5 per cent solution contains 0.005 c. c. of tricresol. The above facts have been experimentally confirmed in this laboratory.

As phenol is not used as a preservative in this country for diphtheria antitoxic serums, preference being given by manufacturers to tricresol, we inoculate mice subcutaneously with 1 c. c. of their serum. Mice so inoculated sometimes show trembling and other manifestations of tricresol poisoning, which shows that the serums contain very



Potency test for Blank's Diph.antitoxin #G4 - B721 opus 6

Date labeled Return after Sept.10-1905.

Date tested 11-2-04

Character of package Syringe

Amount of contents 4 c.c.

No. of units labeled 1.000

= 250 units per cc.

LABEL

Purchased by T.I. at W.A.W. in Phila. Pa. Oct. 15 1904

Physical appearance Opalescent

Odor of tricresol

0.5 cc planted in 100 cc. bouillon: result = Sterile

1.0 cc subcutaneously into mouse: = No effect

1.5 cc intraperitoneally into guinea pig: = No effect

Tested	10-2-04	for	200	units	per cc	G.P.	no.	642	Result	-	nil
"	"	"	225	"	"	"	"	643	"	-	"
"	"	"	250	"	"	"	"	644	"	-	"
	"	"	300	"	"	"	"	645	"		-Died 3 days 16 hrs

FIG. 17.—Illustrating record used for testing the potency and purity of diphtheria antitoxin bought on the open market.

nearly this percentage of tricresol. As the serums practically never contain an excess of preservatives, these inoculations into mice serve as a further test for tetanus and other poisons.

Finally, all that is left of the serum is injected into the peritoneum of a guinea pig in order to discover the presence of any virulent substance of a bacterial or toxic nature.

These tests are repeated with sufficient frequency to establish the fact that the serums made by each licensed manufacturer sold upon the market are pure and potent.

Tables found very useful for diluting the serum in making these tests follow:

TABLE FOR DILUTING SERUMS 100 TO 500 UNITS PER CUBIC CENTIMETER.

First dilution.

1+9=1:10 1 c. c.=0.1

Second dilution.

1 of 1st dil.—

+ 9=1:100	1 c. c.=0.01 or $\frac{1}{100}$=	100 units per c. c.
+10=1:110	1 c. c.=0.00999 or $\frac{1}{110}$=	110 units per c. c.
+11=1:120	1 c. c.=0.00833 or $\frac{1}{120}$=	120 units per c. c.
+12=1:130	1 c. c.=0.00769 or $\frac{1}{130}$=	130 units per c. c.
+13=1:140	1 c. c.=0.00714 or $\frac{1}{140}$=	140 units per c. c.
+14=1:150	1 c. c.=0.00666 or $\frac{1}{150}$=	150 units per c. c.
+15=1:160	1 c. c.=0.00625 or $\frac{1}{160}$=	160 units per c. c.
+16=1:170	1 c. c.=0.00588 or $\frac{1}{170}$=	170 units per c. c.
+17=1:180	1 c. c.=0.00555 or $\frac{1}{180}$=	180 units per c. c.
+18=1:190	1 c. c.=0.00526 or $\frac{1}{190}$=	190 units per c. c.
+19=1:200	1 c. c.=0.005 or $\frac{1}{200}$=	200 units per c. c.
+20=1:210	1 c. c.=0.00476 or $\frac{1}{210}$=	210 units per c. c.
+21=1:220	1 c. c.=0.00454 or $\frac{1}{220}$=	220 units per c. c.
+22=1:230	1 c. c.=0.00434 or $\frac{1}{230}$=	230 units per c. c.
+23=1:240	1 c. c.=0.00416 or $\frac{1}{240}$=	240 units per c. c.
+24=1:250	1 c. c.=0.004 or $\frac{1}{250}$=	250 units per c. c.
+25=1:260	1 c. c.=0.00384 or $\frac{1}{260}$=	260 units per c. c.
+26=1:270	1 c. c.=0.0037 or $\frac{1}{270}$=	270 units per c. c.
+27=1:280	1 c. c.=0.00357 or $\frac{1}{280}$=	280 units per c. c.
+28=1:290	1 c. c.=0.00345 or $\frac{1}{290}$=	290 units per c. c.

TABLE FOR DILUTING SERUMS 100 TO 500 UNITS PER CUBIC CENTIMETER—Continued.

Second dilution—Continued.

1 of 1st dil.—

+29=1:300	1 c. c.=0.00333 or $\frac{1}{300}$=	300 units per c. c.
+30=1:310	1 c. c.=0.00322 or $\frac{1}{310}$=	310 units per c. c.
+31=1:320	1 c. c.=0.00312 or $\frac{1}{320}$=	320 units per c. c.
+32=1:330	1 c. c.=0.00303 or $\frac{1}{330}$=	330 units per c. c.
+33=1:340	1 c. c.=0.00294 or $\frac{1}{340}$=	340 units per c. c.
+34=1:350	1 c. c.=0.00285 or $\frac{1}{350}$=	350 units per c. c.
+35=1:360	1 c. c.=0.00277 or $\frac{1}{360}$=	360 units per c. c.
+36=1:370	1 c. c.=0.0027 or $\frac{1}{370}$=	370 units per c. c.
+37=1:380	1 c. c.=0.00263 or $\frac{1}{380}$=	380 units per c. c.
+38=1:390	1 c. c.=0.00256 or $\frac{1}{390}$=	390 units per c. c.
+39=1:400	1 c. c.=0.0025 or $\frac{1}{400}$=	400 units per c. c.
+40=1:410	1 c. c.=0.00244 or $\frac{1}{410}$=	410 units per c. c.
+41=1:420	1 c. c.=0.00238 or $\frac{1}{420}$=	420 units per c. c.
+42=1:430	1 c. c.=0.00232 or $\frac{1}{430}$=	430 units per c. c.
+43=1:440	1 c. c.=0.00227 or $\frac{1}{440}$=	440 units per c. c.
+44=1:450	1 c. c.=0.00222 or $\frac{1}{450}$=	450 units per c. c.
+45=1:460	1 c. c.=0.00217 or $\frac{1}{460}$=	460 units per c. c.
+46=1:470	1 c. c.=0.00213 or $\frac{1}{470}$=	470 units per c. c.
+47=1:480	1 c. c.=0.00208 or $\frac{1}{480}$=	480 units per c. c.
+48=1:490	1 c. c.=0.00204 or $\frac{1}{490}$=	490 units per c. c.
+49=1:500	1 c. c.=0.002 or $\frac{1}{500}$=	500 units per c. c.

TABLE FOR DILUTING SERUMS 200 TO 1,000 UNITS PER CUBIC CENTIMETER.

First dilution.

1+19=1:20 1 c. c.=0.05.

Second dilution.

1 of 1st dil.—

+ 9=1:200	1 c. c.=0.005 or $\frac{1}{200}$ =	200 units per c. c.
+10=1:220	1 c. c.=0.00454 or $\frac{1}{220}$ =	220 units per c. c.
+11=1:240	1 c. c.=0.00416 or $\frac{1}{240}$ =	240 units per c. c.
+12=1:260	1 c. c.=0.00384 or $\frac{1}{260}$ =	260 units per c. c.
+13=1:280	1 c. c.=0.00357 or $\frac{1}{280}$ =	280 units per c. c.

TABLE FOR DILUTING SERUMS 200 TO 1,000 UNITS PER CUBIC CENTIMETER—Continued.

Second dilution—Continued.

1 of 1st dil.—

+14=1:300	1 c. c.=0.00333 or	$\frac{1}{300}$ =	300 units per c. c.
+15=1:320	1 c. c.=0.00312 or	$\frac{1}{320}$ =	320 units per c. c.
+16=1:340	1 c. c.=0.00294 or	$\frac{1}{340}$ =	340 units per c. c.
+17=1:360	1 c. c.=0.00277 or	$\frac{1}{360}$ =	360 units per c. c.
+18=1:380	1 c. c.=0.00263 or	$\frac{1}{380}$ =	380 units per c. c.
+19=1:400	1 c. c.=0.0025 or	$\frac{1}{400}$ =	400 units per c. c.
+20=1:420	1 c. c.=0.00238 or	$\frac{1}{420}$ =	420 units per c. c.
+21=1:440	1 c. c.=0.00227 or	$\frac{1}{440}$ =	440 units per c. c.
+22=1:460	1 c. c.=0.00217 or	$\frac{1}{460}$ =	460 units per c. c.
+23=1:480	1 c. c.=0.00208 or	$\frac{1}{480}$ =	480 units per c. c.
+24=1:500	1 c. c.=0.002 or	$\frac{1}{500}$ =	500 units per c. c.
+25=1:520	1 c. c.=0.00192 or	$\frac{1}{520}$ =	520 units per c. c.
+26=1:540	1 c. c.=0.00185 or	$\frac{1}{540}$ =	540 units per c. c.
+27=1:560	1 c. c.=0.00182 or	$\frac{1}{560}$ =	560 units per c. c.
+28=1:580	1 c. c.=0.00172 or	$\frac{1}{580}$ =	580 units per c. c.
+29=1:600	1 c. c.=0.00166 or	$\frac{1}{600}$ =	600 units per c. c.
+30=1:620	1 c. c.=0.00161 or	$\frac{1}{620}$ =	620 units per c. c.
+31=1:640	1 c. c.=0.00156 or	$\frac{1}{640}$ =	640 units per c. c.
+32=1:660	1 c. c.=0.00151 or	$\frac{1}{660}$ =	660 units per c. c.
+33=1:680	1 c. c.=0.00147 or	$\frac{1}{680}$ =	680 units per c. c.
+34=1:700	1 c. c.=0.00143 or	$\frac{1}{700}$ =	700 units per c. c.
+35=1:720	1 c. c.=0.00139 or	$\frac{1}{720}$ =	720 units per c. c.
+36=1:740	1 c. c.=0.00135 or	$\frac{1}{740}$ =	740 units per c. c.
+37=1:760	1 c. c.=0.00131 or	$\frac{1}{760}$ =	760 units per c. c.
+38=1:780	1 c. c.=0.00128 or	$\frac{1}{780}$ =	780 units per c. c.
+39=1:800	1 c. c.=0.00125 or	$\frac{1}{800}$ =	800 units per c. c.
+40=1:820	1 c. c.=0.00122 or	$\frac{1}{820}$ =	820 units per c. c.
+41=1:840	1 c. c.=0.00119 or	$\frac{1}{840}$ =	840 units per c. c.
+42=1:860	1 c. c.=0.00116 or	$\frac{1}{860}$ =	860 units per c. c.
+43=1:880	1 c. c.=0.00113 or	$\frac{1}{880}$ =	880 units per c. c.
+44=1:900	1 c. c.=0.00111 or	$\frac{1}{900}$ =	900 units per c. c.
+45=1:920	1 c. c.=0.00109 or	$\frac{1}{920}$ =	920 units per c. c.
+46=1:940	1 c. c.=0.00106 or	$\frac{1}{940}$ =	940 units per c. c.
+47=1:960	1 c. c.=0.00104 or	$\frac{1}{960}$ =	960 units per c. c.
+48=1:980	1 c. c.=0.00102 or	$\frac{1}{980}$ =	980 units per c. c.
+49=1:1,000	1 c. c.=0.001 or	$\frac{1}{1000}$ =	1,000 units per c. c.

SERUM ANTIDIPHTHERICUM IN THE PHARMACOPŒIA.

The next edition of the United States Pharmacopœia, being the eighth decennial revision, 1900, which is to appear shortly, will contain an antitoxic serum for the first time. The serum will be known officially as antidiphtheric serum or *Serum antidiphthericum* and the unit will be recognized as that approved or established by the United States Public Health and Marine-Hospital Service.

The official text, which has been kindly furnished by Professor Remington in advance, will be as follows:

SERUM ANTIDIPHTHERICUM

ANTIDIPHTHERIC SERUM DIPHTHERIA ANTITOXIN

A fluid separated from the coagulated blood of a horse *Equus caballus* Linné, immunized through the inoculation of diphtheric toxin. It should be kept in sealed glass containers, in a dark place, at temperatures between 4.5° and 15° C. (40° and 59° F.).

> A yellowish or yellowish-brown, transparent or slightly turbid liquid, odorless or having a slight odor, due to the presence of the antiseptic used as a preservative.
>
> Specific gravity: 1.025 to 1.040 at 25° C. (77° F.).
>
> Antidiphtheric Serum gradually loses its power, the loss in one year varying between 10 per cent. and 30 per cent. Each container should be furnished with a label or statement, giving the strength of the Antidiphtheric Serum, expressed in antitoxic units, the name and percentage by volume of the antiseptic used for the preservation of the liquid (if such be used), the date when the Antidiphtheric Serum was last tested, and the date beyond which it will not have the strength indicated on the label or statement.
>
> The standard of strength, expressed in units of antitoxic power, should be that approved or established by the United States Public Health and Marine-Hospital Service.

Average dose.—3000 units.

Immunizing dose for well persons.—500 units.

The only other pharmacopœia officially recognizing diphtheria antitoxin is the Arzneibuch für das Deutsche Reich (Pharmacopœa Germanica), fourth edition, 1900. The following is a translation of the German text:

SERUM ANTIDIPHTHERICUM—DIPHTHERIE-HEILSERUM.

The blood serum of horses which have been immunized against the diphtheria poison. It is allowed to be sold in commerce by authorized manufacturers only after it has been tested by the Königlich preussische Institut für experimentelle Therapie zu Frankfurt a. M. These tests include the strength of the serum in immunity units (Immunisirungseinheiten = I. E.), the freedom from bacteria, and the percentages of substances (phenol or trikresol), which have been added as preservatives.

It is offered for sale in both the liquid and solid forms.

Liquid and solid antidiphtheric serum are purveyed only in bottles which are officially sealed. Upon the label is stated the place of manufacture, the antitoxic value of one cubic centimeter, and the total contents of the bottle; also the control number and the date on which the official control was exercised. These bottles are placed in light-proof packages containing the same label; the lead seals show an eagle or a lion on the obverse and the total number of immunity units (I. E.) contained in the bottle on the reverse.

The liquid antidiphtheric serum is a yellow, clear fluid having at most a very slight precipitate; it has the odor of the preservative used. It is put up in bottles of various form and color, the contents of which contain from 100 to 3,000 I. E. The bottles most frequently used are those containing:

No. 0 = 200 I. E.
No. I = 600 I. E. (or 500 I. E.)
No. II = 1,000 I. E.
No. III = 1,500 I. E.

Antidiphtheric serum which contains more than 300 I. E. in 1 c. c. is considered a high grade serum.

The solid antidiphtheric serum is a dried serum of high grade, and contains at least 5,000 I. E. in 1 gram. It contains no antiseptic or other added substance. It appears as a yellowish-white powder, soluble in ten parts of water, giving a solution which, in color and appearance, resembles the corresponding liquid antidiphtheric serum.

Single doses of from 250 to 1,000 I. E., and containing 2 to 6 c. c., are placed in white glass-stoppered bottles. The solution should be made with sterile water in the original bottle, using 1 c. c. for each 250 I. E., and should be prepared fresh each time. The solution should be clear except for little albuminous particles, and should be used from the original bottle.

Serum with a marked permanent cloudiness or an abundant precipitate is not allowed to be dispensed by the apothecaries; also serums with certain control numbers which have been recalled.

TO BE KEPT IN A COOL PLACE AND PROTECTED FROM THE LIGHT.

THE OFFICIAL METHODS USED IN GERMANY FOR TESTING DIPHTHERIA TOXINE AND ANTITOXIN.

The German methods which follow have been taken largely from E. Marx: Die Experimentelle Diagnostik Serumtherapie und Prophylaxe der Infectionskrankheiten, 1902.

In Germany the governmental control of diphtheritic serum is effected through the imperial act of December 31, 1894, which forbids unrestricted commerce in diphtheritic serum. The sale and traffic in diphtheritic serum is controlled by the Königliches Institut für experimentelle Therapie zu Frankfurt a. M., which is designated as the official place of examination for the Empire. Apothecaries are allowed to deal only in sera which are thus controlled.

This control naturally begins at the place of manufacture and consists of an inspection to assure the use of none but healthy horses in the production of serum. Official regulations are prescribed concerning the animals, the injection of toxines, and the drawing of blood from horses.

When a manufacturer has several liters (3 to 10) of tested serum this quantity is brought to the Government officer (known as the Abnahmebeamte) at the place of its manufacture. At the same time exact data upon the history of the particular serum must be given. The Abnahmebeamte takes a small quantity of the serum, places 3 to 5 c. c. in each of six or eight bottles, which he guards with a lead seal, and, attaching the necessary labels, sends the bottles to the testing station at Frankfurt a. M. The serum that remains at the factory is placed by the Abnahmebeamte under official lock and key until he receives the results of the tests.

The official testing of diphtheria antitoxin was started in Germany at the Institut für Infectionskrankheiten, of Berlin, February 20, 1895.[a]

A serum is tested—

(*a*) To see that it is harmless, and

(*b*) For its antitoxic value.

1. It must be clear or, at most, have a very slight precipitate.
2. It must be free from bacteriæ, as tested by bacteriological methods.
3. It must not contain more than 0.5 per cent of carbolic acid or cresol.

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[a] Ehrlich: Die staatliche controle des diphtherieserums. Ber. klin. woch., v. 33 (20), May 18, 1896, pp. 441–443.

The institute for testing the serum does not determine in its official tests the exact antitoxic value of sera sent it, but determines by means of the L+ dose of the test poison whether the serum contains at least the value claimed for it by the manufacturer. The testing for the sterility of the sera is done in accordance with well known bacteriologic rules, by making aërobic and anaërobic agar and bouillon cultures.

In order to determine the amount of disinfectants added to the serum white mice are used. They are inoculated subcutaneously with 0.5 c. c. of the serum. Solutions of carbolic acid containing more than 0.5 per cent unerringly cause, in this amount, the death of the animal. Finally, guinea pigs are inoculated with 10 c. c. of the serum, and they must remain well.

If the serum has complied with all the official tests, it is considered reliable, and, after due notification of the fact, it is immediately bottled in the presence of the local official. It is the duty of this officer to seal each bottle with his lead seal. His control is a guaranty of the procedure of this part of the process, and he is responsible for the proper labeling of the bottles that are sold on the market.

A further control is carried out in this way: Of every test number in the testing institute six and twenty-four months afterwards the serum is again tested to determine whether it contains the stated value and has not lost strength. Also, a number of hospitals are commissioned, from time to time, as they use the bottles of serum, to test them for sterility. Sera that lose strength or show bacterial contamination are withdrawn from the market.

From experience gained in his work, Ehrlich suggested the following amendments to the instructions for the testing of diphtheria antitoxin, which were officially promulgated by the minister of education, etc., on March 29, 1897:

1. A powdered serum protected against moisture and oxygen serves as the measure by which the strengths of other serums are determined. This powdered serum is preserved in exactly weighed quantities in special vacuum tubes. At this time in the Institut the tubes contain 2 grams each of the dry powdered antitoxin, which have the value of 1,700 times normal strength (1,700 facher Stärke).

2. The serum is dissolved in a mixture of equal parts of a 10 per cent solution of sodium chlorid and glycerin, this mixture favoring the stability of the antitoxin. Every three months a tube is opened and a new solution made. At present the contents of the tubes preserved in the Institut are dissolved in 200 c. c. of the above-mentioned mixture, which gives a test serum containing 17 units in each cubic centimeter.

3. The present test dose of toxin is determined by means of an

immunity unit which is contained in 1 c. c. of the test serum mentioned in paragraph 2, diluted 17 times. This quantity (1 c. c.) of serum is mixed with increasing amounts of the diphtheria poison, and the mixture inoculated into a series of animals, in order to determine with the greatest possible accuracy the least amount sufficient to cause the death of the animals in the first four days. This quantity of the diphtheria poison is then taken as the quantity to be used for the present test dose. Varying quantities of toxin are again added to the same quantity of serum (viz, 1 unit) in order to determine a second Limit, namely, that quantity of diphtheria poison which must be added to the serum in order that the poison will be exactly neutralized as indicated by inoculating the mixture into guinea pigs.

4. The estimation of the strength of a diphtheria antitoxin is accomplished by means of a test dose of the poison (see paragraph 3) in this way: The particular test dose—for example, 0.355 c. c.—which is the present test dose of the toxin determined at the Institut, is added to or mixed with 4 c. c. of a mixture containing one unit of the abovementioned serum.

As the test dose of the poison is determined against 1 c. c. of a normal serum, or against 4 c. c. of a one-fourth normal serum, consequently in testing a serum of (supposed) x strength the test dose of poison is added to 4 c. c of a $\frac{1}{4x}$ dilution of that serum, or against 4 c. c. of a $\frac{1}{400}$ dilution of a serum of (supposed) one hundred times normal strength.

5. The mixture obtained is injected subcutaneously into guinea pigs weighing 250 to 280 grams. If the animals tested by two members of the Institut die within the first four days, then that particular serum does not contain the strength claimed for it. If the animals die within the fifth or sixth day, then the serum is exactly on the limits of admissibility, and in order to prevent the necessity of soon recalling the serum from the market it is recommended to the manufacturer to add 5 to 10 per cent. Indurations that appear in the test animals are not taken into consideration in this test.

An autopsy is performed upon animals that die, to determine whether complicating diseases are present, such as tuberculosis, pseudotuberculosis, or pneumonia, which may have rendered the animals more susceptible.

6. Either the liquid or solid poison may be used for testing as long as the two limits defined in paragraph 3 have been properly determined and the difference between the two limits does not exceed 15 × MLD. Liquid poisons preserved with toluol must be used only under the following conditions:

(1) If after prolonged examination it is known that the stability of the test dose is constant and (2) when the test dose does not exceed

1 c. c. The observations on the quality of the test dose follow in the succeeding paragraphs.[a]

7. The test poisons are, when liquid, to be examined at least once a month upon culture media for sterility.

8. The test dose of poison is to be redetermined every six weeks by means of the test dose of serum in such a way that both the test dose of the poison and its neutralizing point may be determined anew. If in these redeterminations a considerable weakening of the test dose develops, then the poison must be considered as being in dissolution and should be replaced by a new one.

9. The attention of manufacturers must be brought to the fact that the test poison in small quantities is very apt to decompose; particularly a very short exposure to light will cause an appreciable weakening. It is therefore recommended that they obtain every three weeks a new poison from the Institut.

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[a] According to my observations, to carry out the test with exactness it is not necessary for the test dose to contain a maximum amount of poisonous affinities. From the viewpoint of technique, old weakened poisons preserved with toluol are preferred, because after the weakening has taken place they will preserve their value for years.

BIBLIOGRAPHY OF ARTICLES CONSULTED IN WRITING THIS BULLETIN.

Aronson, Hans.

1893. Experimentelle Untersuchungen über Diphtherie und die immunisirende Substanz des Blutserums. <Berl. klin. Wchnschr., v. 30, pp. 592; 625.

1894. Weitere Untersuchungen über Diphtherie und das Diphtherie-Antitoxin. <Ibidem, v. 31, pp. 356; 425; 453.

Arrhenius, Svante; & Madsen, Thorvald.

1902. Physical chemistry applied to the study of toxins and antitoxins. <State Serum Inst., Denmark, III, pp. 1–87.

On the molecular weight of diphtheria toxin. A preliminary note. <State Serum Inst., Denmark, IV, pp. 1–7.

1904. Toxines et antitoxines le poison diphthérique. <Bull. Acad. roy. sci. et let., Danemark, no. 4, pp. 269–305.

Behring, [E.].

1890. Untersuchungen über das Zustandekommen der Diphtherie-Immunität bei Thieren. <Deutsche med. Wchnschr., Leipz. u. Berl., v. 16 (49–50), pp. 1145–1148.

1892. Die Blutserumtherapie bei Diphtherie und Tetanus. Einleitung. <Ztschr. f. Hyg., Leipz., v. 12, pp. 1–9.

Ueber Immunisirung und Heilung von Versuchsthieren beim Tetanus. <Ibidem, v. 12, pp. 45–57.

1893. Zur Behandlung der Diphtherie mit Diphtherieheilserum. <Deutsche med. Wchnschr., Leipz. u. Berl., v. 19 (23), pp. 543–547.

1894. Antitoxisch wirkende Desinfectionsmittel. Allgemeine Bemerkungen über antitoxische Mittel. <Ibidem, v. 20 (8), pp. 169–171.

Behring, [E.] Boer; [O.] & Kossel, [H.].

1893. Zur Behandlung diphtheriekranker Menschen mit Diphtherieheilserum. <Ibidem, v. 19 (17–18), pp. 389–393; 415–418.

Behring [E.]; & Boer, O.

1894. Ueber die quantitative Bestimmung von Diptherieantitoxin-Lösungen. <Ibidem, v. 20 (21), pp. 453–454.

Behring [E.] & Kitasato [E.].

1890. Ueber das Zustandekommen der Diphtherie-Immunität, und der Tetanus-Immunität bei Thieren. <Ibidem, v. 16 (49), pp. 1113–1114.

Behring [E.]; & Nissen, F.

1890. Ueber bacterienfeindliche Eigenschaften verschiedener Blutserumarten. Ein Beitrag zur Immunitätsfrage. <Ztschr. f. Hyg., Leipz., v. 8, pp. 412–433.

Behring [E.]; & Wernicke.

1892. Ueber Immunisirung und Heilung von Versuchsthieren bei der Diphtherie. <Ibidem, v. 12, pp. 10–44.

BORDET, JULES.

1896. Sur le mode d'action des sérums préventifs. <Ann. de l'Inst. Pasteur, Par., v. 10 (4), pp. 193–219.

1898. Sur l'agglutination et la dissolution des globules rouges par le sérum d'animaux injectés de sang défibriné. <Ibidem, v. 12 (10), pp. 288–295.

1903. Sur le mode d'action des antitoxines sur les toxines. <Ibidem, v. 17 (3), pp. 161–186.

BUCHNER, H.

1893. Ueber Bacteriengifte und Gegengifte. <Münch. med. Wchnschr., v. 40 (24–25), pp. 449–452; 480–483.

1901. Sind die Alexine einfache oder complexe Körper? <Berl. klin. Wchnschr., v. 38 (33), pp. 854–857.

COBBETT, L.

1899. The origin of antitoxin. Is it present in the blood of some normal animals. <Lancet, Lond., v. 2, Aug. 5, pp. 332–337.

DANYSZ, JEAN.

1899. Contribution à l'étude de l'immunité. Propriétés des mélanges des toxines avec leurs antitoxines. Constitution des toxines. <Ann. de l'Inst. Pasteur, Par., v. 13 (7), pp. 581–595, 2 figs.

DIEUDONNÉ, ADOLF.

1903. Immunität, Schutzimpfung und Serumtherapie; Zusammenfassende Übersicht über Immunitätslehre. Leipsig. J. A. Barth. 176 pp. 8°.

DREYER, GEORGES.

1900. Sur l'immunisation à l'aide des toxones. <XIII^{e} Cong. internat. de méd., Paris, Sec. de bact. et de parasitol., pp. 45–47.

1901. Ueber die Grenzen der Wirkung des Diphtherieheilserums gegenüber den Toxonen des Diphtheriegiftes. <Ztschr. f. Hyg., Leipz., v. 37, pp. 268–274.

1904. Immunity: Agglutination. <Brit. M. J., Lond., v. 2, Sept. 10 (2280), pp. 564–567.

DREYER, GEORGES; & MADSEN, THORVALD.

1901. Ueber Immunisirung mit den Toxinen des Diphtheriegiftes. <Ztschr. f. Hyg., Leipz., v. 37, pp. 250–267.

1902. Studies on diphtheria toxin. <State Serum Inst., Denmark, V, pp. 1–6.

v. DUNGERN.

1900. Beiträge zur Immunitätslehre. <Münch. med. Wchnschr., v. 47 (20), pp. 677–680 (28), pp. 962–965.

1904. Beitrag zur Kenntniss der Bindungsverhältnisse bei der Vereinigung von Diphtheriegift und Antiserum. <Deutsche med. Wchnschr., Leipz. u. Berl., v. 30 (8–9), pp. 275–277; 310–312.

EHRLICH, PAUL.

1891. Experimentelle Untersuchungen über Immunität. I. Ueber Ricin. <Ibidem, v. 17 (32), pp. 976–979.

Experimentelle Untersuchungen über Immunität. II. Ueber Abrin. <Ibidem, v. 17 (44), pp. 1218–1219.

1896. Die staatliche Controle des Diphtherieserums. <Berl. klin. Wchnschr., v. 33 (20), pp. 441–443.

1897. Zur Kenntniss der Antitoxinwirkung. <Fortschr. d. Med., Berl., v. 15 (3), pp. 41–43.

Die Werthbemessung des Diphtherieheilserums und deren theoretische Grundlagen. <Klin. Jahrb., Jena, v. 6 (2), pp. 299–326.

Ehrlich, Paul—Continued.

1898. Ueber die Constitution des Diphtheriegiftes. <Deutsche med. Wchnschr., Leipz. u. Berl., v. 24 (38), pp. 597 690.

1900. Ueber Toxine und Antitoxine. Ibidem, v. 26 (33), p. 189 L.

Croonian lecture. On immunity with special reference to cell life. <Proc. Royal Soc. Lond., v. 66, pp. 424–448, pls. 6–7.

1901. Schlussbetrachtungen. Speciellen Path. u. Ther., Wien, v. 8, pp. 1–25, 1. pl.

Ueber Toxine und Antitoxine. Therapie der Gegenwart. Berl., Urban & Schwarzenberg, pp. 1–32.

1902. Ueber die Complementophilen Gruppen der Amboceptoren. <Berl.klin. Wchnschr., v. 39 (25), pp. 585–587.

Ueber die Beziehungen von chemischer Constitution, Vertheilung und pharmakologischer Wirkung. <Internat. Beitr. z. inneren Med., v. 1, pp. 646–679.

1903. Toxin und Antitoxin. Entgegnung auf Grubers Replik. <Münch. med. Wchnschr. v. 50 (52), pp. 2295–2297.

Betrachtungen über den Mechanismus der Ambozeptorwirkung und seine teleologische Bedeutung. <Festschr. z. 60. Geburtstage v. Rob. Koch, Jena, pp. 509–526.

Toxin und Antitoxin. Entgegnung auf den neuesten Angriff Grubers. <Münch. med. Wchnschr. v. 50 (33–34), pp. 1428–1432; 1465–1469.

Ueber die Giftcomponenten des Diphtherietoxins. <Berl. klin. Wchnschr. v. 40 (35–37), pp. 793–797; 825–829; 848–851.

1904. Herter lecture††: Physical chemistry *versus* biology in the doctrines of immunity. (Abstract), 8 pp.

Gesammelte Arbeiten zur Immunitätsforschung. Berlin, August Hirschwald, 776 pp.

Ehrlich, P., Kossel, H., & Wassermann, A.

1894. Ueber Gewinnung und Verwendung des Diphtherieheilserums. Deutsche med. Wchnschr., Leipz. u. Berl., v. 20 (16), pp. 353–355.

Ehrlich, P.; & Kossel, H.

1894. Ueber die Anwendung des Diphtherieantitoxins. <Ztschr. f. Hyg., Leipz., v. 17, pp. 486–488.

Ehrlich, (Paul); & Morgenroth.

1901. Ueber Hämolysine. <Berl. klin. Wchnschr. v. 38 (10), pp. 251–257, 6 figs.

1904. Wirkung und Entstehung der aktiven Stoffe im Serum nach der Seitenkettentheorie. Handb. d. Path. Mikroorg., W. Kolle & A. Wassermann, Jena, v. 4, 1st part, pp. 430–451.

Ehrlich, P.; & Sachs, H.

1902. Ueber die Vielheit der Complemente des Serums. <Berl. klin. Wchnschr., v. 39 (14–15), pp. 297–299; 335–338.

Ueber den Mechanismus der Amboceptorenwirkung. <Ibidem, v. 39 (21), pp. 492–496.

Flexner, Simon; & Noguchi, Hideyo.

1902. Snake venom in relation to haemolysis, bacteriolysis, and toxicity. <J. Exper. Med., N. York, v. 6, pp. 277–301.

Gruber, (Max).

1901. Zur Theorie der Antikörper. <Münch. med. Wchnschr., v. 48 (46 u. 48), pp. 1827–1830; 1924–1926.

GRUBER, M.; & v. PIRQUET, CL.
1903. Toxin und Antitoxin. <Münch. med. Wchnschr. v. 50 (28–29), pp. 1193–1196; 1259–1263; 7 figs.

HUNT, REID.
1905. Toxins. <Gould's Am. Year Book of Med. and Surg., p. 649.

KYES [PRESTON].
1903. Ueber die Isolirung von Schlangengift-Lecithiden. <Berl. klin. Wchnschr., v. 40 (42–43), pp. 956–962; 982–984.

KYES, PRESTON; & SACHS, HANS.
1903. Zur Kenntniss der Cobragift activirenden Substanzen. <Ibidem, v. 40 (2–4), pp. 21–23; 57–66; 82–85.

LANCET COMMISSION.
1896. Report of the Lancet special commission on the relative strengths of diphtheria antitoxic serums. <Lancet, Lond., v. 2, pp. 182–195.

MACÉ, E,
1901. Traité pratique de bactériologie. 4. éd. Paris, J. B. Baillière & Sons, 1195 pp., 338 figs.

MADSEN, TH(ORVALD).
1897. Zur Biologie des Diphtheriebacillus. <Ztschr. f. Hyg., Leipz., v. 26, pp. 157–192, 1 pl., 5 figs.
Ueber Messung der Stärke des antidiphtherischen Serums. <Ibidem, v. 24, pp. 425–442.
1899. Ueber Tetanolysin. <Ibidem, v. 32, pp. 214–245.
Ueber Heilversuche im Reagensglas. <Ibidem, v. 32, pp. 239–245.
La constitution du poison diphthérique. Première partie. <Ann. de l'Inst. Pasteur, Par., v. 13 (7), pp. 568–580, 3 figs.
La constitution du poison dipththérique. Deuxième partie. <Ibidem, v. 13 (11), pp. 801–832, 8 figs.
1900. Sur les toxones. <XIII^e Cong. internat. de méd., Paris. Sec. de bact. et de parasitol, pp 40–45.
1903. La constitution du poison diphthérique. <Centralbl. f. Bakteriol. (etc.), 1. Abt., Jena, v. 34, pp. 630–641, 4 figs.
1904. Toxins and antitoxins. <Brit. M. J., v. 2, Sept. 10 (2280), pp. 567–574.

MADSEN, TH(ORVALD); & WALBURN, L.
1904. Toxines et antitoxines de la ricine et de l'antiricine. <Bull. de l'Acad. royale d. sci. et d. let. d. Danemark, Copenhague, no. 3, pp. 81–103.

MARKL.
1902. Ueber Hemmung der Hämolyse durch Salze. <Ztschr. f. Hyg., Leipz., v. 39, pp. 86–92.

MARTIN, C. J.
1904. Immunity: snake venoms. <Brit. M. J., v. 2, Sept. 10 (2280), pp. 574–577.

MARTIN, C. J.; & CHERRY, THOMAS.
1898. The nature of the antagonism between toxins and antitoxins. <Proc. Royal Soc. Lond., v. 63, pp. 420–432.

MARTIN, LOUIS.
1898. Production de la toxine dipthtérique. <Ann. de l'Inst. Pasteur, Par., v. 12, pp. 26–48.

MARX (E.).

1901. Experimentelle Untersuchungen über die Beziehung zwischen dem Gehalt an Immunitätseinheiten und dem schützenden und heilenden Werth der Diphtherieheilsera. <Ztschr. f. Hyg., Leipz., v. 38, pp. 372–385.

1902. Die experimentelle Diagnostik, Serumtherapie und Prophylaxe der Infectionskrankheiten. Berlin, 296 pp., 1 fig., 2 pls.

MCFARLAND, JOSEPH.

1904. A text-book upon the pathological bacteria for students of medicine and physicians. Philadelphia, W. B. Saunders & Company. 4th ed., 629 + 16 pp., 153 figs.

MELTZER, S. J.; & AUER, JOHN.

1905. On the rate of absorption from intramuscular tissue. <Am. Med., v. 9 (102), p. 75. (Reported proceedings of the Soc. Exper. Biol. and Med.)

METCHNIKOFF, ÉLIE.

1901. L'immunité dans les maladies infectieuses. Paris, Masson & Cie., 600 pp., 45 figs.

MICHAELIS, LEONOR.

1904. Ueber die Gültigkeit des Massenwirkungsgesetzes bei der Reaction zwischen Toxin und Antitoxin. <Biochem. Centralbl., v. 3 (1), pp. 1–12.

MORGENROTH, J.

1899. Ueber den Antikörper des Labenzyms. <Centralbl. f. Bakteriol. (etc.), Jena, v. 26 (11–12), pp. 349–359.

1902. Ueber die Bindung hämolytischer Ambozeptoren. <Münch. med. Wchnschr., v. 50 (2), pp. 61–62.

1903. Ueber Gruber's Kälteeinwand gegen die Ambozeptortheorie. <Wien. klin. Wchnschr., v. 16 (43), pp. 1183–1184.

1903–4. Komplementablenkung durch hämolytischer Ambozeptoren. <Centralbl. f. Bakteriol. (etc.), Jena, v. 35, pp. 501–505.

1904. Untersuchungen über die Bindung von Diphtherietoxin und Anti-Toxin, zugleich ein Beitrag zur Kenntniss der Constitution des Diphtheriegiftes. <Ztschr. f. Hyg., Leipz., v. 48, pp. 177–238.

Untersuchungen über die Bindung von Diphtherietoxin und Antitoxin, sowie über die Constitution des Diphtheriegiftes. <Berl. klin. Wchnschr., v. 41, pp. 526–530.

Ambozeptortheoric und Kälteversuch. <Wien. klin. Wchnschr., v. 17, p. 126.

MUIR, ROBERT.

1904. Immunity: Haemolytic serums. <Brit. M. J., Lond., v. 2, Sept. 10 (2280), pp. 577–580.

NEISSER, MAX.

1897. Zur Differentialdiagnose des Diphtheriebacillus. <Ztschr. f. Hyg., Leipz., v. 24, pp. 443–469.

NICOLLE [M.].

1896. Préparation de la toxine diphtérique. <Ann. de l'Inst. Pasteur. Par., v. 10, pp. 333–334.

OPPENHEIMER, CARL.

1903. Die Bakteriengifte. Handbuch Pathogenen Mikroorganismen, bei Kolle, W. u. Wassermann, A. Jena, v. 1, ch. 7, pp. 344–379.

1904. Toxine und Antitoxine. Jena, Gustav Fischer, 227 pp.

PARK, W. H.; & WILLIAMS, A. W.

1896. The production of diphtheria toxin. <J. exper. med., N. York, v. 1 (1), pp. 164–185.

RITCHIE, JAMES.

1902. A review of current theories regarding immunity. <J. Hyg., Cambridge, v. 2 (2, 3, 4), pp. 215–250; 251–285; 452–454.

1904. Immunity: A general review. <Brit. M. J., Lond., v. 2, Sept. 10 (2280), pp. 557–560.

ROUX, E.

1900. Mesure de l'activité des sérums. <X. Internat. Cong. f. Hyg. u. Demographie, pp. 21–26.

ROUX, E.; & MARTIN, L.

1894. Contribution à l'étude de la diphthérie (sérum-thérapie). <Ann. de l'Inst. Pasteur, Par., v. 8 (9), pp. 609–639.

ROUX, E.; & YERSIN, A.

1888. Contribution à l'étude de la diphthérie. <Ibidem, v. 2 (12), pp. 629–661.

1889. Contribution à l'étude de la diphthérie. (2^{e} mémoire.) <Ibidem, v. 3 (6), pp. 273–288.

1890. Contribution à l'étude de la diphthérie. (3^{e} mémoire.) <Ibidem, v. 4 (7), pp. 385–426.

SACHS, HANS.

1901. Immunisierungsversuche mit immunikörperbaladenen Erythrocyten. <Centralbl. f. Bakteriol., Jena, v. 30 (13), pp. 491–494.

1902. Giebt es einheitliche Alexinwirkungen? <Berl. klin. Wchnschr., v. 39 (9–10), pp. 181–183; 216–218.

SALMON, D. E.; & SMITH, T.

1884–6. On a new method of producing immunity from contagious diseases. <Proc. Biol. Soc. Wash., v. 3, pp. 29–33.

1887. Experiments on the production of immunity by the hypodermic injection of sterilized cultures. (Abstract.) <Tr. Internat. M. Cong., Wash., v. 3, pp. 403–407.

SALOMONSEN, C. J.; & MADSEN, THORVALD.

1897. Recherches sur la marche de l'immunisation active contre la diphthérie. <Ann. de l'Inst. Pasteur, Par.. v. 11 (4), pp. 315–331.

1898. Sur la reproduction de la substance antitoxique après de fortes saignées. <Ibidem, v. 12 (11), pp. 763–773, 4 figs.

1899. Recherches sur la marche de l'immunisation active contre la diphthérie. 2^{me} mémoire. <Ibidem, v. 13 (3), pp. 262–272, 5 figs.

SMITH, THEOBALD.

1896. The conditions which influence the appearance of toxin in cultures of diphtheria bacillus. <Tr. Ass. Am. Physicians, Phila., v. 11, pp. 37–61.

1898. The toxin of diphtheria and its antitoxin. <Bost. M. & S. J., v. 139 (7–8), pp. 157; 192.

1899. The relation of dextrose to the production of toxin in bouillon cultures of the diphtheria bacillus. <J. Exper. Med., N. York, v. 4 (3–4), pp. 373–397.

1900. The antitoxin unit in diphtheria. <J. Bost. Soc. Med. Sci., v. 5 (1), pp. 1–11, 1 fig.

1905. Degrees of susceptibility to diphtheria toxin among guinea pigs. Transmission from parent to offspring. <J. M. Research, Bost., v. 13 (3), p. 341.

Smith, T.; & Walker, E. L.

1896. A comparative study of the toxin production of diphtheria bacilli. <Rep. Bd. Health Mass., Bost., pp. 649–672.

1897–8. A comparative study of the toxin production of diphtheria bacilli. <J. Bost. Soc. Med. Sci., v. 2, pp. 12–15.

Spronck, C. H. H.

1895. Sur les conditions dont dépend la production du poison dans les cultures diphtériques. Moyen simple de préparer une toxine très active. <Ann. de l'Inst. Pasteur, Par., v. 9, pp. 758–765.

1898. Préparation de la toxine diphtérique; suppression de l'emploi de la viande. <Ibidem, v. 12, pp. 701–704.

Van Calcar, R. P.

1904. Ueber die Constitution des Diphtheriegiftes. <Berl. klin. Wchnschr., v. 41 (39), pp. 1028–1031, 1 fig.

Vaughan, Victor C.

1904. Further studies of the intracellular bacterial toxins. <J. Am. Med. Ass., Chicago, v. 43 (10), pp. 643–647.

Vaughan, Victor C.; & Novy, Frederick G.

1902. Cellular toxins or the chemical factors in the causation of disease. Phila. and N. York, Lea Brothers & Company. 4th ed., rev. and enlarged, 495 pp., 2 figs., 1 table.

Wassermann, A.

1894. Ueber Concentrirung der Diphtherieantitoxine aus der Milch immunisirter Thiere. <Ztschr. f. Hyg., Leipz., v. 18, pp. 235–238.

1896. Experimentelle Untersuchungen über einige theoretische Punkte der Immunitätslehre. <Ibidem, v. 22, pp. 263–313.

1898. Weitere Mittheilungen über Seitenketten-Immunität. <Berl. klin. Wchnschr., v. 35, pp. 209–211.

Ueber eine neue Art von künstlicher Immunität. <Ibidem, v. 35, p. 4.

1901. Experimentelle Beiträge zur Kenntniss der natürlichen und künstlichen Immunität. <Ztschr. f. Hyg., Leipz., v. 37, pp. 173–202.

1902. Hämolysine, Cytotoxine und Präcipitine. <Klin. Vorträge, Leipz. (331), pp. 339–384.

1903. Experimentelle Beiträge zur Frage der aktiven Immunisierung des Menschen. <Festschr. z. 60. Geburtst. v. Rob. Koch, Jena, pp. 527–540.

1904. A discussion on several new points concerning the theory and practice of immunity. <N. York M. J. (etc.), v. 80 (16), pp. 721–725.

Die Grundzüge der Lehre von der Immunität und Serumtherapie. <Ztschr. f. ärztl. Fortbild., Jena, v. 1, pp. 17; 41.

Wassermann, A.; & Bruck, C.

1904. Ueber die Wirkungsweisse der Antitoxine im lebenden Organismus. <Deutsche med. Wchnschr., Leipz. u. Berl., v. 30, pp. 764–766.

Wassermann, A.; & Ostertag, R.

1904. Ueber polyvalente (multipartiale) Sera mit besonderer Berücksichtigung der Immunität gegenüber den Erregern der Schweineseuche. <Ztschr. f. Hyg., Leipz., v. 47, pp. 416–427.

Welch, William H.

1894. Bacteriological investigations of diphtheria in the United States. (A report in behalf of the American committee on diphtheria to the eighth international congress of hygiene and demography held in Budapest September 1–9, 1894.) <Am. J. Med. Soc. Phila., v. 108 (4), n. s., pp. 427–461.

1902. The Huxley lecture on recent studies of immunity with special reference to their bearing on pathology. <Bull. Johns Hopkins Hosp., Balto., v. 13 (141), pp. 285–299.

Wright, A. E.

1904. Immunity: opsonines. <Brit. med. journ., Sep. 10, 1904, p. 582.

O

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